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PODARIUM (*PODARIA*) – A CONTROVERSIAL BOTANICAL TERM

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Abstract: Succulent plants and especially cacti require specific botanical terms for their morphological description. *Podarium* is one such term, used mostly to designate the spine-bearing formations in cacti and thorny succulent euphorbias. The analysis of specialized literature reveals its use by various authors with different meanings. The term is present in dictionaries or specialised glossaries only in the last 30 years, and the definitions are quite varied. The synonymous terms used over time in different languages show that there is no unity regarding the definition of the term, but also regarding the way in which the formation it defines is understood.

Key words: botanical terms, Cactaceae family, *Euphorbia*, Lemaire, spine-shield, tubercle.

Introduction

Botanical terminology, like all specialized terminology, has certain characteristics that differentiate it from common language. In the general sense, scientific terms are characterized by clarity and lack of ambiguity. These aspects are not always obvious and are not understood by all specialists in the same way [SLISKO & DYKSTRA, 1997]. Often terms evolve during their use in one or more fields, and sometimes they are rejected for various reasons (there are accepted synonyms, they are not clearly represented, etc.).

There is an ongoing concern to standardize botanical terms and make their use uniform. In some botanical families, the problem of terms has given rise to numerous efforts, due to the morphological peculiarities of some species, e.g. Poaceae [JACQUES-FÉLIX, 1972], Cucurbitaceae [NESOM, 2012], Loganiaceae [CONN, 1995].

Cacti are also among the plants with a novel structure, so it was only natural that there would be a variety of theories and terms to explain it. The need for terminological uniformity in the treatment of the Cactaceae family is eloquently highlighted by a comment on "Flora Argentina", in which STUCKERT (1899) draws attention to the fact that the author names the cactus family **Cactáceas**, but also **Cácteas**, which constitutes "una falta de uniformidad"/ a lack of uniformity.

Podarium (pl. *podaria*) is a botanical term used to describe structure specific to cacti species, but also other succulents. We consider it deserves attention for several reasons: it is little known (in some languages it is not used and there is no corresponding term), it designates a formation present in few plants, it has a precise "moment of birth" and, last but not least, it appears very rarely in dictionaries or glossaries of specialized terms.

In this paper we wish to draw attention both to the botanical term *podarium* and to the inconsistencies, misunderstandings and chaos created around the use of terms, out of the desire

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to describe (especially in cactus and euphorbia families) similar morphological formations. At the same time, we point out that in the case of the two mentioned families, cacti and euphorbias, respectively, the botanical terms "spines" and "thorns", especially in German literature, are often mistaken ("stacheln" = "dornen" = "thorns", for both cacti and euphorbia, while for roses, for example, it is "stacheln" = "thorns", "prickles", in English).

Moreover, the description of some formations that appeared as a result of the convergent evolution of the characters, in different plant families, on different continents, as well as the ignorance of some authors on all the descriptive works published to the date, have resulted over the years in the accumulation of many inconsistencies and confusion in the use of terms.

Material and methods

In order to clarify the term *podarium*, numerous bibliographic references have been consulted (most of them available online) looking for aspects regarding the chronological use of the term since its first documented use, its presence in works written in widely spoken languages (English, German, French, Spanish, Italian) and / or the presence of the translation of this term in relation (especially of synonymy) with other terms.

The analysis of the bibliographic material is not exhaustive, as it is impossible to verify all the works that refer to or mention the term *podarium*.

For a more accurate analysis, the authors have turned to some classic works in the field of terminology. From these works we selected two definitions to represent initial working hypotheses:

"The term ... is a linguistic symbol that is assigned to one or more concepts, which are defined by related concepts" [FELBER, 1984].

"[The term] is a lexical unit that consists of one or more words that represent a concept within a field" [BESSÉ & al. 1997].

Results and discutions

Podarium (plural, *podaria*) is a botanical term with limited use, which in the Romanian scientific literature is practically unknown.

Its etymology is from the Greek language: *podos* – foot; it has been taken up in various languages as follows:

French: sg. *podaire* / pl. *podaires*; English: sg. *podarium* / pl. *podaria*; German: sg. *podarium* / pl. *podarien*; Spanish: sg. *podario* / pl. *podarios*; Latin: sg. nominative *podarium* / pl. dative *podariis*.

The frequency of appearance of this term in specialized works, especially regarding the Cactaceae family, is closely correlated with the number of cacti and succulent enthusiasts.

Charles Lemaire and the need for proper botanical terms

The history of this term begins in 1858, when Lemaire uses the term "podaires" (French) as a synonym for "teeth" in the description of the species *Euphorbia hermentiana* [LEMAIRE, 1858a], but also to designate the protuberances present on *Pelecyphora aselliformis* [LEMAIRE, 1858b]. In fact, to designate the protuberances specific to cacti, Lemaire proposes another term – *cÿrtome*. He shows that the protuberances of *Pelecyphora aselliformis*, called *podaires*, are different from those of some *Echinopsids*, hence called cÿrtomes, and both are equivalent to Salm-Dyck's *tubercules* (tubercles).

Lemaire proposed these new botanical terms because he was dissatisfied with those in use at the time: *tubercle* (sg.) / *tubercles* (pl.) [ENGELMANN, 1856; HOOKER, 1839a] and *mammilla* (sg.) / *mammillae* (pl.) in English [HOOKER, 1839b]; *tuberculum* (sg.) / *tuberculis* (pl.) [DE CANDOLLE, 1829; HOOKER, 1839a] and *mammilla* (sg.) / *mammillas* (pl.) in Latin [HAWORTH, 1819; HOOKER, 1839b; WALPERS, 1845-46]; *tubercule* (sg.) / *tubercules* (pl.) [LEMAIRE, 1841-1847], *mamelon* (sg.) / *mamelons* (pl.) [LEMAIRE, 1857b, 1858c], *dents* (pl.) [LEMAIRE, 1857a] and *gibbosité* (sg.) / *gibbosités* (pl.) [LABOURET, 1853; LEMAIRE, 1857a, b, 1858d] in French.

In the same article Lemaire promises to demonstrate that the *mamilla* (v. *tuberculus*) represents a true petiole, and the term(s) which is / are not proper must be replaced by something else suitable: "…verbo isti non apto aliud omnino congruens erat substituendum – podarium (ποδαρίον, parvus pes)"/ for this unsuitable word, another completely suitable word was to be substituted – podarium.

In the following years Lemaire constantly uses the term *podaire* in the description of the species presented in the journal coordinated by Verschaffelt, including in the Latin diagnosis: *podariis* [LEMAIRE, 1860a], *cyrtomis* [LEMAIRE, 1859, 1860b] respectively. Probably the consecration of the term *podaire* and the Latinized form *podarium* are due to the botanist Pierre Edmond BOISSIER (1862) who uses the term proposed by the horticulturist Lemaire in the volume dedicated to the Euphorbiaceae family in De Candolle's Prodromus.

In the same year LEMAIRE (1862) reviews Boissier's work and notes with satisfaction the use of the term proposed four years ago. Thus Lemaire believes that BOISSIER (1862, 1866) adopted the term because it is "convenient for the describer/descriptor"; in addition, it was "missing from the nomenclature and expresses well what needs to be pointed out". He also describes the term podaire (podarium) as "the extension in the form of gibbosity which, in fleshy euphorbias, carries the leaf, its stipule (thorn) and the flowers in the axil or a secondary branch that constantly aborts". Lemaire likens it to the protuberances of *Mammillaria*, *Echinocactus*, *Echinocereus* within the Cactaceae family.

In 1868 LEMAIRE publishes a comprehensive paper on cacti, on which occasion he brings numerous clarifications on the subject, as he had promised in 1858. He points out once more the analogy of the organs designated by *podarium* in succulent euphorbias and cacti. He also highlights the fact that in the species of cacti with areoles, this "curious and strange" organ is actually the metamorphosed petiole and even states that *podarium* is "synonymous with petiole".

We can speculate that Lemaire's proposal to replace the terms may have also been influenced by the peculiarities of the French language. Specifically, the protuberances specific to cacti are referred to in English as *tubercles*, different from the *tuber* which generally refers to the thickened parts of the modified stems. This differentiation is not found in the French language, where the term tubercule designates both types of structures.

Here is the definition of the term in the DICTIONARY of the FRENCH ACADEMY, ed. 6, in the year 1835: "Tubercule (terme de jardinage). Excroissance en forme de bosse qui survient à une feuille, à une racine, à une plante. Il se dit plus particulièrement de celles qui se forment à la racine de certaines plantes alimentaires. *Les pommes de terre*, *les topinambours sont des tubercules*" / Tuber (garden term). Bump-shaped growth that occurs on a leaf, a root, a plant. It is said more particularly of those which are formed at the root of certain food plants. Potatoes, Jerusalem artichokes are tubers.

To better understand Lemaire's proposal we will quote some definitions from the specialized dictionaries of the time. These seem restrictive and do not refer to the interesting structures specific to cacti and fleshy euphorbias:

Tubercle, *tuberculum* (lat. a pimple): a small wart-like excrescence. Synonym: a form of Apothecium [HENSLOW, 1840]; succulent expansion of certain roots [COOKE, 1862]. *Tuber*. An annual thickened subterranean stem (…). When very small it is called *tubercle* [HOBLYN, 1850].

Mamilla (a little teat). Little granular prominences on the surface of certain pollen [HENSLOW, 1840]; granular prominences on pollen-grain [COOKE, 1862]; a term applied to a surface which is studded over with prominences which are smooth, thick, and rounded, like a teat [HOBLYN, 1850].

JOURDAN'S polyglot dictionary (1837) is not of much help either:

Tubercule (fr.), *tuberculum* (lat.), *Hockerchen* (germ.), the definition of this term is very extensive, but we only note that it is applied to lichens, algae, or the thickened, starchy parts of plants.

Mamelon (fr.), *mamilla* (lat.), *warze* (germ.), *nipple* (engl.). Protuberance arrondie, qui fait sailie au milieu d' une surface quelconque. / Rounded protrusion, which protrudes from the middle of any surface. In the botanical field, the author exemplifies this term only for "the top of some mushrooms that have a conical hat".

Only in the case of the adjective "*mamillaire* (*mamillaris*, *mamilla*, *mamelle*) = which resembles a nipple", we learn that we encounter this aspect in *Euphorbia mamillaris* "which bears tubercles in the shape of nipples".

It is also worth mentioning that the term Warze (Germ.) is equivalent to *verrue* (Fr.) / *verruca* (Lat.) and *papille* (Fr.) / *papilla* (Lat.).

Lemaire was trying to propose a term to replace the phrases used by the authors to express as correctly as possible the appearance of the protuberances specific to cacti or fleshy euphorbias: „tubercules mammiformes" [LABOURET, 1853], "plantae mamillato-tuberculatae" [ENGELMANN, 1857], „costis mammillosis" [PFEIFFER, 1845], "tuberculis mamilliformibus elongates" [LEMAIRE, 1855].

We pointed out that Lemaire actually proposed two terms which he states are necessary in treating the genera of the family Cactaceae. The free protuberances, true petioles, characteristic of the genus *Mammillaria* are called *podaire* (podarium), while the confluent protuberances with the adjacent tissue, present in genera such *as Cereus*, *Pilocereus*, *Echinocereus*, *Aporocactus* represent *cyrtôme*.

As early as 1829 DE CANDOLLE argues that a distinction must be made between *tubercles* and *mammillas*, as he considers the long protuberances from species of the genus *Mammillaria* to be true leaves. This differentiation is specified by other authors, but it is very obvious in the bilingual article published by PFEIFFER & OTTO (1843). The equivalent terms in Latin, German and French are different, depending on the genus to which the plants belong:

Echinocactus: *tuberculis* (Latin) – *Höckern* (German) – *tubercules* (French).

Mammillaria: *mammillis* (Latin) – *Warzen* (German) – *mamelons* (French).

HAWORTH (1819) and ENGELMANN (1856) prefer the term *tubercle/tuberculis* to describe most cacti known at the time.

The term *podarium* – a chronological perspective

In the period following the publication of De Candolle's work, leading biologists of the time took up the term in their works to describe spiny species of *Euphorbia*, especially in the Latin diagnosis [HOOKER, 1865; GROENLAND, 1866; SCHWEINFURTH, 1866; BAYLEY, 1888; NORTON, 1900; ENGLER, 1902; BERGER, 1899, 1902], including in works published in Japan [HAYATA, 1904].

In a few years the term is adopted in German – *podarium* (sg.) / *podarien* (pl.). It is used in papers to designate important formations in the differentiation of species or as a diagnostic character in the keys to determine the species of *Euphorbia* analyzed [PAX, 1895, 1905a,b; BERGER, 1905a; VIERHAPPER, 1907]. After the 1930s, the term is used more and more frequently in German-language specialist journals [NEL, 1933, 1935-1936; WERDERMANN & MANSFELD, 1935; STEPHAN, 1937].

BERGER is an author with numerous works in German who adopted the term proposed by Lemaire in the description of the analyzed species, most often synonymous with leaf base. In the valuable work in which he analyzes succulent euphorbias, he uses several terms to describe the same characteristic formation: *podarium* / *podarien*, synonymous alternately with *Blattbasen* but also *Blattkissen*, as well as *Höcker*, and in 1905 also with *Warzen*. It is worth recalling his clarification regarding the fact that "Die Blattbasen (*Podarien*) laufen bei den allermeisten dieser *Diacanthium*-Arten in fortlaufende Längsrippen oder Kanten zusammen./ In the vast majority of these *Diacanthium* species, the leaf bases (*podaria*) converge in continuous longitudinal ribs or edges.

From his numerous articles on succulent species, it can be seen that BERGER predominantly uses the term warzen (1905c) to describe cacti, only in a work from 1926 does he use the term *podarien* (oder Blattkissen) / *podaria* (or cushion).

Berger's proposed classification of the group of succulent Euphorbias is largely taken up by PAX in a collective volume on the vegetation of the earth (1921). Pax also uses the term *podarien / podarium*, most likely in Berger's sense (*Podarien = Blattbasen*), only in describing species of section *Diacanthium*. About this he states: "perhaps the most difficult group of relatives of the whole genus, as herbarium material is scarce and photographs of the growth conditions are mostly lacking", and characteristic is the presence of "thorny podaria, which merge into rib-like ledges or edges".

An exception to the use of the term is in the case of subsect. Tetracanthae, where he uses the term *Fußstücke* in the key. In the presentation of the subsection, it states that its species "ausgezeichnet durch vier, paarweise zu-sammengehörige Dornen am Podarium" / are distinguished by four pairs of spines on the podarium.

Croizat is one of the authors who frequently uses the term *podarium*, in different variants (English, French, Latin). From the analysis of his works in French [CROIZAT, 1938a] and English [CROIZAT, 1939] we deduce how Croizat understands the structure of the spiny protuberances of succulent euphorbias: *podaire* couverte des écussons épineux / podaria with spine-shields. Probably these works are among the first in which the English term spine-shields appears as the horny part covering the apex of the protuberance.

In 1939 Croizat explains the term *podarium* by *tooth to the wing or angle,* recalling the expression "dents (podaires!)" used by Lemaire, and few years latter [CROIZAT, 1942] the author defined podarium as "decurrent succulent petioles". We observe that Croizat emphasizes the constant character of this formation, so that it can be considered a diagnosis character.

BOITEAU (1947) shows that the thornless euphorbias from the Cape region, South Africa (*Euphorbia bupleurifolia*, *E. pubiglans*) show "des *mamelons* formés par les *podaires* rudimentaires" (*mammilles* formed by rudimentary *podaria*), different from *podaires* specific to the subsection *Diacanthium*. In this case the term *mamelons* probably does not have the same meaning as in Lemaire and is among the few instances when it is used to describe the stems of some species of the genus *Euphorbia*. BAILLON (1858) uses the same term both to describe the portion of the leaf base that persists on the stems of fleshy euphorbias and to designate structural elements in the organization of the gynoecium in species of this genus.

From the analysis of the available bibliographic data, it seems that until around the 1940s the term *podarium* with its various forms appears mainly in articles related to the genus *Euphorbia*. That is probably why some authors [WHITE & al. 1941] define the term *podarium* as "A raised foot or stand; the term is used in many writings on the *Euphorbiae*". On the other hand, about the *tubercle* he states that "... designate the swollen base of a leaf or leaf rudiment, such as is found in many different forms on the stems and branches of most of the highly succulent species of *Euphorbiae*".

We will further look at issues regarding the use of the term podarium in articles about cacti.

BERGER has numerous articles on cacti; only in 1926 does he use the term *Podarium* (pl. *Podarien*) to explain the way some species develop, then in 1929 he prefers the term warzen, which he mentions is synonymous with *podarium* (*Hökker*).

It is worth emphasizing that Lemaire's works are cited by authors from Latin America, that is, the homeland of cacti. In the 1937 paper, BRAVO HOLLIS, a renowned Mexican botanist, uses the term *tuberculos* (Spanish) in the species description; she cites Lemaire with a paper from 1853, before he proposed the two terms. Lemaire was previously also cited by OCHOTERENA (1922) with the work "Les Cactées", but without mentioning the year. It is difficult to say whether the author was aware of the proposed terms, which ARECHAVALETA mentioned as early as 1905, in the volume of the Flora of Uruguay, where he uses *tuberculos* or *mamilas* in the description of the species.

BUXBAUM is the one who has an indisputable contribution in clarifying the aspects related to the morphology, ontogeny and anatomy of cacti. There are many articles in which he refers to the morpho-anatomical features of cacti (1956), and the contribution to the volume edited by Krainz [BUXBAUM, 1956-1960] is defining.

It is very likely that his volume, published in English [BUXBAUM, 1950], facilitated the access to its valuable information to a much larger number of authors and also enhanced the use of the term *podarium*. The work includes very detailed explanations on the formations specific to cacti (areoles, protuberances, cephalium), but also on the term *podarium*. About this term Buxbaum states that it represents an enlargement of leaf base and neighboring regions of the stem, which together take the form of a protuberance (tubercle). In fact, *podarium* designates the juvenile stage of these formations, which either grow alone in an outgrowth (tubercle) or converge among others in a vertical direction forming ribs.

The richly illustrated work allows the understanding of the formation of the protuberance that is present in cacti, but also in other succulent plants (it only exemplifies euphorbias).

The 1940-1955 period is characterized by the appearance of Buxbaum's works [BUXBAUM, 1949], which has extremely valuable contributions in clarifying some cacti-specific terms. In the works that appeared later, in the description of the representatives of the Cactaceae family different terms are used depending on the authors: *tubercle* [TAYLOR, 1979], *tuberculos* [KIESLING, 1984]; *podarium* [DONALD, 1971, 1976; PEUKERT, 1977], *podariis* / *podario* [BRAVO HOLLIS, 1956; MEYRÁN, 1956]; *mamelon* and *gibbosités* [GAY, 1954]; *mamelones* [CASTELLANOS, 1962], *podario* or *tuberculo* [BRAVO HOLLIS, 1978], *tubercles* or *podaria* [BRUHN & HOLMSTEDT, 1973]; *warzen* and *Hökker* [BACKEBERG, 1958].

In order to clarify the relationship between the terms protuberances (tubercles) and ribs, commonly used in the description of cacti, Zimmerman [BUTTERWORTH & al. 2002] recommended in 1985 the use of the term *podarium*, suggesting that in reality the ribs are series

of *podaria* joined together. Protuberances represent free or distinct podaria. This terminology allows for intermediation between ribs and protuberances.

GIBSON & NOBEL (1986) proposed a different view of the term. Thus from an anatomical point of view the podaria are similar to the parts of the stem which surround the base of the leaf and are simple swellings of the stem over which the leaves and their modified axillary buds (areoles) rise. They show that the *podaria* are "bases in the sense of a pedestal for the leaves, not the basal portion of the leaves themselves". Extensive cell division in the *podarium* contributes to the formation of tubercles.

After 1990, the term *tubercle / tuberculos* is frequently used in texts about cacti [MONTES & al. 1997], most often specifying that it is synonymous with *podarium*.

A unanimous acceptance of the term *podarium* (among other terms) is not to be found even after the year 2000. TAYLOR (2000), in his doctoral thesis highlights the need to clarify some terms used in the text and offers brief explanations/definitions for areoles, glochids, pericarpe, flower-tube and *podaria*.

"*podaria* (sing. *podarium*) are the swellings often subtending areoles that represent the points of attachment of leaves or bracts that have been lost, or almost lost, in the course of evolution of the highly succulent habit"

The comments of DICHT & LÜTHY (2005), which illustrate the difficulties caused by the lack of terms or their different understanding (depending on the authors), are to be understood in the same context:

"…the areole essentially consists of three different organs: the spiniferous part, the groove, and the axil. In most cactus literature the expression areole is used only for the spine-bearing part. For the whole areole, another word, e.g. *podarium*, should be used. For clarity and consistency with former publications, we describe the three parts of the podarium separately as areole (spine-bearing part), groove and axil."

The term podarium in works relating to euphorbias and other succulents

Efforts to clarify terminology specific to cacti have no equivalent in the situation of the genus *Euphorbia* (or other succulents). At least until 1977, when S. CARTER publishes a short note in which she analyzes the advantages of using the term *podarium* to describe succulent euphorbias. Careful analysis of the information in this text in conjunction with data from previous works reveals confusion and diverse interpretations of this aspect.

Although the history of the term podarium is already starting to be rich, the statement that until 1962 "no author questioned its validity" seems inexplicable. Because, as we have shown, as early as 1950 Buxbaum clarified aspects of morphology and organogenesis related to the structures called *podarium*, also including references to the genus *Euphorbia*.

The author recognizes (only then) the accuracy of the term "podarium" compared to "scutellum", which she specifies already has four uses, and persisting in using it as a synonym for podarium can lead to "confusion". The term *scutellum* is very rarely used in relation to the appearance of fleshy euphorbias, we find it at COSSON (1874) in the Latin diagnosis of the species of the section *Diacanthium*. Three years before he designated however the formations present in *E. resinifera* with the term *podariis* [COSSON, 1871], corresponding in French description with "coussinets" / cushions. The mention of the term *scutellum* highlights a confusion that seems to have very old roots, perhaps even in the works of Boissier.

Podarium for Lemaire means a small foot (parvus pes – Latin), while *scutellum* means shield, which makes us associate it with the term spine-shield. The latter designates the horny pad on which the thorns usually develop in some species of *Euphorbia*.

If we analyze the works published over time, it becomes obvious that the term *podarium* was used with two different meanings:

1. as a protuberance similar to that of cacti, therefore equivalent to *tubercle*, *mammilla* or *mamelon* [BERGER, 1905a,b; CHEVALIER, 1933; WHITE & al. 1941; URSCH & LEANDRI, 1954],

2. as a horny pad covering the protuberance, in this case designating a formation specific to spiny euphorbias [COSSON, 1871; PAX, 1909; BRUCE & al. 1951; KEAY, 1955; BALLY, 1973; BALLY & CARTER, 1974; VERDUS, 1973; LEACH, 1976a,b] called *spine-shield* (English) or *coussinet foliare* (French).

To understand where this difference in interpretation comes from, we will return to the works of Boissier. Thus in Prodromus (1862) BOISSIER uses the term *podarium / podaria* within the group of succulent euphorbias and shows that for the definition of the section *Diacanthium* "Pulvini foliorum prominentes in tuberculos elevatos (podaria Ch. Lemaire)" is characteristic. Within the subsection *Biaculeatae podaria* represents a differentiating character: "Podaria basi distincta" versus "podaria in costas confluentia".

Various phrases appear in the description of the species:

Euphorbia drupifera: "ramis ob podaria subdissita" / branches divided by podaria; *E. canariensis*: "ramisque ... calloso-tuberculatis tuberculis fuscis" / branches with ... callous-tuberculate brown tubercles; *E. caput medusae*: "ramos ... podariis depressis obtuse carinatis basi decurrentibus" / branches ... with podaria obtusely carinated from the base; *E. scopoliana*: "costis subverticalibus e podariis mamillaeformibus subdistinctis conicis acutis constantibus" / subvertical ribs from the nipples-shaped podaria subtended by acute cones.

In the case of the thornless species (*E. drupifera*, *E. caput medusae* and *E. scopoliana*), the term *podarium* (*podariis* – declination in Latin) seems to have the meaning of *tubercle*, while in the spiny species *E. canariensis* the formations are called *tuberculis/tubercles*, and the term *podarium* is missing from the diagnosis.

In the French work Icones Euphorbiarum (1866), BOISSIER shows that in the *Diacanthium* section "...les coussinets des feuilles se groupent pour former des tubercules coniques tantôt distincts, tantôt réunis en côtes verticales ou spirales"/ the cushions of the leaves are grouped to form conical tubercles sometimes distinct, sometimes united in vertical ribs or spirals.

Comparing the Latin and French texts it seems that actually for Boissier *podarium* is equivalent to *coussinets des feuilles*, so *spine-shield* in English. This may mean that he took over the term proposed by Lemaire, but with a different meaning.

A similar phrase – "coussinets écarté" (spread cushions) is used by CHEVALIER (1951) to describe the spiny succulent euphorbias of the Congo. It is difficult to say whether the expression is synonymous with *podaria* or *spine-shields*.

Phrases such as "Kegelförmigen *Podarien*" / conical *podaria* [BERGER, 1899], "*Podarien* verlängent, kleine flachkegelige Warzen bildend" / *podaria* elongated, forming small flat-conical warts [BERGER, 1905a] or "Die Warzen (*Podarien*) der Stengel" / the warts (*podaria*) of the stems [BERGER, 1905b] show that Berger used *podarium/podarien* sensu Lemaire. On the other hand, although Pax mostly takes Berger's classification (which he cites) for the *Diacanthium* section, he uses the term *podarium/podariis* sensu Boissier.

The inconsistency of the understanding of the term podarium persists and is all the more obvious when we encounter it within the works of the same author. Thus GILBERT (1987) in a paper in which he presents two new geophytic species of *Euphorbia* compares the characteristics of the representatives of the subgenera *Euphorbia* and *Lacanthis*. He shows that

one of the important differences is the way the spines occur (subgen. *Euphorbia*) or not (subgen. *Lacanthis*), on the "horny pad around the insertion of the leaf at the tip of the prominence (podarium)" (spines borne on clearly defined horny spine-shield round leaf insertion at tip of tubercle (podarium)).

He also claims that a subsection should be recognized for euphorbias of the subgenus *Diacanthium* native to Madagascar and gives an example of the unusual characteristic of *E. corniculata* where "the spine-shield are unusually large and almost entirely cover the *podaria* on which they are mounted".

In the same year, in the description of the species *Euphorbia heterospina* together with SUSAN C., GILBERT [CARTER & GILBERT, 1987] uses the term *podaria/podariis* with the meaning of *spine-shields*.

The analysis of later published works shows that very frequently the term *podaria* (Latin or English) is translated by *spine-shield* (English) [CARTER, 1987; BUDDENSIEK & al. 2005; KILIAN & al. 2006; MALPURE & al. 2016]. WEBER & al. (2020) prefer the term *podaria* to name the formations present in *Euphorbia poisonii*, called *tubercles* by BROWN (1911).

The terms *podaria/podarium* also appear sporadically in works (predominantly of taxonomy) on succulent species from other genera and families: Apocynaceae [MEVE, 1997; MÜLLER & ALBERS, 2004], *Beiselia* (Burseraceae) [FORMAN & al. 1989], Burseraceae [EGGLI, 2002], Didiereaceae [RAUH & DINKLAGE 1979], Fouquieraceae [ROWLEY, 2002a], *Cotyledon* [POELLNITZ, 1942], *Othonna* (Asteraceae) [ROWLEY, 2002b], *Peperomia* (Piperaceae) [NYFFELER & ROWLEY, 2002].

Rauh shows that the organization of the species of Didiereaceae is similar to that of primitive cacti, specifying that *podaria* are present in the genus *Didierea*, similar to the genus *Leuchtenbergia* [RAUH, 1983]. They also consider brachyblasts homologous to areoles. If we extend this homology to the *Euphorbia* species of the *Diacanthium* section, the *podarium* is homologous with long-shoots and protuberances, and the spine-shield with brachyblasts and areoles. FRANCK (2016) talks about the difference between long-shoots and short-shoots in *Harrisia* species where he shows that "the green photosynthetic stems have indeterminate long-shoots which contain numerous short-shoots. A cactus short-shoot is referred to as an areole". A summary of these analogies is shown in Table 1.

Tabel 1. Homologous structures in *Euphorbia*, *Didierea* and *Leuchtenbergia* genus

Homologous structures	**Genus**		
	Euphorbia (*Diacanthium*)	*Didierea*	*Leuchtenbergia*
	podarium	long-shoots	protuberance
	spine-shield	brachyblasts	areole

The difficulty of naming formations specific to succulent plants with a single phrase is obvious: RAUH (1979b) uses the formulation "Mamillenartige bildengen" / nipple-like formations, while a decade before FRIEDRICH (1968) used the phrases "höckerig-warzigen oder kurz stiftförmingen Auswüchsen" / bumpy-warty or short pin-shaped outgrowths with the meaning *podarien / podaria* in the description of the genus *Cotyledon*. In the same context, we mention Chevalier's 1933 work, which uses four different terms to describe African succulent species: *protuberances* (*E. sudanica*), *tubercule* (*E. unispina*, *E. tellieri*, *E. sapini*), *ecusson* (*E. darbandensis*) and *mamelons* (in the determination key – section *Diacanthium*).

JACOBSEN (1954) uses the terms *Basen der Blätter*, *Blattpolster*, *Blattkissen*, *Warzen* and *podarien* to describe the same formation in different species of the genus *Euphorbia*. Only in the description of group 19 (section *Florispinae* Haw.) does it indicate the synonymy between *Warzen* and *Podarien*. It is difficult to say whether either term is synonymous with spine-shield.

In the case of the genera *Ocimum* (Lamiaceae) [VOGEL, 1998] and *Polypodium* (Pteridophyta!) [HAGEMANN, 1969] the use of the term *podarium* is disconcerting and requires much wider discussion. In the same context is the statement of HEADS (1994): "The nature of the leaf-base (= leaf-cushion, podarium, soubassement foliaire, etc.) in *Leonohebe* (Plantaginaceae, formerly Scrophulariaceae) is problematic, and has hardly been studied".

Synonymy relationships with other terms/expressions

It is noted that over time, the term *podaria* has been synonymous with different botanical terms or expressions, which do not all have the same meaning: tubers, areoles (in their entirety), the base of the leaves (in the sense mentioned by Buxbaum – the thickened portion of the leaf petiole), horned pad (preferred translation for spine-shields), ridged stem teeth (in the case of *Euphorbia* species), collective term for the protuberances on the surface of cacti (areoles, tubercles).

In a limited number of works, the term *pulvinus*, *cushion* (English), *coussinet* (French) [CHEVALIER, 1951; URSCH & LEANDRI, 1954] appears used in connection with the special formations present in cacti and other succulents.

For an easier understanding of the amplitude of the variation of the terms or expressions used to designate the specific formations discussed so far, the information gathered from the analyzed bibliographic sources [KILIAN & al. 2006; KAPLAN, 2001; CROIZAT, 1942; TROLL & WEBER, 1954; LEMAIRE, 1865; LEACH, 1969; MARNIER-LAPOSTOLLE, 1966; NEUWINGER, 1996; BRAVO-HOLIS, 1978; CALVENTE, 2010; LÜTHY, 1996; LÓPEZ & al. 2015; BRUYNS, 2022] is presented in the Table 2.

We notice that terms such as gonflement, teeth, projection mainly refer to appearance, while areoles, tubercles or spine shields designate a well-defined anatomical structure. From a semantic point of view between these terms and podarium there seems to be a relationship which is illustrated in the image below:

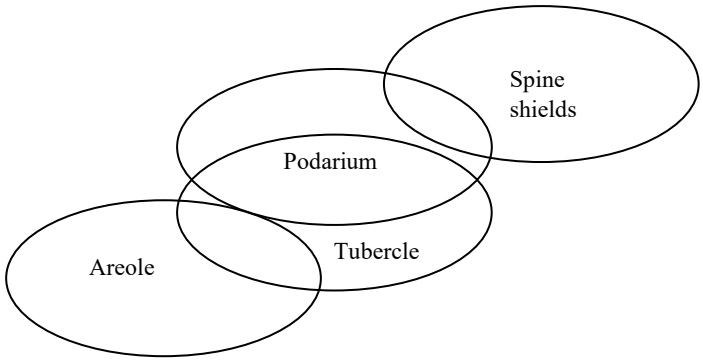

Figure 1. A hypothetical semantic relations between *podarium* and related terms used by various authors.

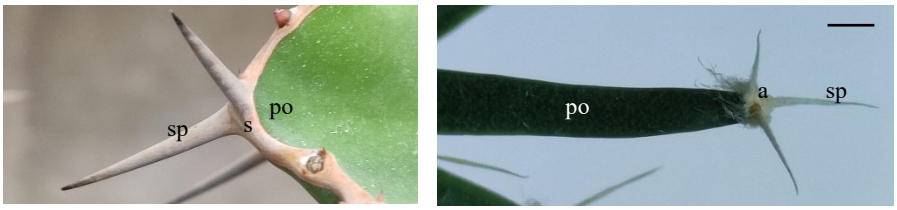

Figure 2. A. *Euphorbia grandicornis*, podarium (po) with spine-shield (s) and spines (sp). B. *Leuchtenbergia principis*, podarium (po) with areole (a) and spines (sp). (Scale 1 cm)

Table 2. Terms and expression synonymous with *podarium* used in botanical references.

Term/expresion sinonymous with *podarium*	**Author/Authors**	**Language**	**Publication year**	**Botanical Family / Genus**
Ampliacao da base foliar	Calvente	Spanish	2010	Cactaceae
Areole (whole)	Dicht & Lüthy	English	2005	Cactaceae
Basen von Blättern/ Blattbasen	Troll & Weber	Deutsch	1954	Cactaceae
	Berger	Deutsch	1907	Cactaceae
Blattpolster	Rauh	Deutsch	1979	*Euphorbia*, Asclepiadaceae (Stapelieen), *Allauadia*
Coussinet (foliaires)	Cosson	French	1871	*Euphorbia*
	Verdus	French	1973	*Euphorbia*
	Ursch & Leandri	French	1954	*Euphorbia*
Cushion	Kaplan	English	2001	Cactaceae
Decurrent succulent petioles	Croizat	English	1942	*Euphorbia*
Dents	Lemaire	French	1858	*Euphorbia*
Ecusson epinier	Marnier-Lapostolle	French	1966	*Euphorbia*
Enlarged leaf pedicel	Rosas-Reinhold & al.	English	2021	Cactaceae
Gaine	Boiteau	French	1947	*Euphorbia*
Gonflement	Groenland	French	1866	*Euphorbia*
Leaf base	Barthlott & Hunt	English	1993	Cactaceae
	Endress & al.	English	2018	Cactaceae
	Kaplan	English	2001	Cactaceae
Mamelons	Kiesling	French	1999	Cactaceae
	Chevalier	French	1933	*Euphorbia*
L' organe pétioliforme	Lemaire	French	1865	*Euphorbia*
Peculiari escrescenze del fusto	Mosti & al.	Italian	2001	Cactaceae
Protuberancia tuberculada	Rivas Rossi	Spanish	1998	Cactaceae
Spine shields	Kilian & al.	English	2006	*Euphorbia*
	Dyer	English	1937	*Euphorbia*
	Keay	English	1955	*Euphorbia*
	Leach	English	1969	*Euphorbia*

Teeth of stems	Croizat	English	1939	*Euphorbia*
Tubercle	Gilbert	English	1987	*Euphorbia*
	Neuwinger	English	1996	*Euphorbia*
	Bruyns	English	2022	*Euphorbia*
Tubercule	Chevalier	French	1933	*Euphorbia*
Tubérculo	Bravo-Holis	Spanish	1978	Cactaceae
	López & al.	Spanish	2015	Cactaceae
Warzen	Berger	Deutsch	1905, 1926	*Euphorbia*
	Lüthy	Deutsch	1996	Cactaceae

When analyzing the data of the Table 2, Figure 1 and Figure 2 it becomes obvious the terms *podarium*, *tubercle*, *areola* and *spine shield* are not synonymous, much less the term *scutellum*.

POC (Plant Ontology Consortium) ruling on the term *podarium*

The POC analysis from November 2011 established the synonymy of the term *podarium* with *tubercle*, which it defined as: "An enlarged leaf base that is fused with adjacent shoot axis tissue".

However, this ruling does not resolve the semantic chaos surrounding the term *podarium*. In the case of the family *Didiereaceae*, the equivalent term is not specified and *tubercle* is not usual, and for the genus *Euphorbia* the relationship with spine-shield is not clarified.

Even in the case of cacti, things are not so simple. The question of the relationship of the terms *tubercle-mammilla* needs to be clarified. If we analyze over time how the two terms have been used we often find confusing aspects.

Thus in the general description of the botanical characters of cacti GUILLAUMIN (1933) uses the term *mamelons*, which he shows are fleshy and probably represent branches. However, in the description of most of the genera, he uses the term *tubercule*, the same term used to describe the ornaments present on the seeds. A few decades later (1984) KIESLING uses the term *tuberculos* to describe cacti, then in 1996 he uses *mamelones* and *podarios* [KIESLING, 1996].

There are several examples of works in Spanish that highlight the confusion caused by the loose use of terms.

Thus FLORES (2005) seems to use the terms *mamillas* and *tubérculos* (Spanish) sensu DeCandolle, but the fact that both appear in the description of different species of the genus *Coryphantha* contradicts this assumption. In addition, the term *protuberancias/protuberances* present in the description of some species of *Ancistrocactus* and *Astrophytum* makes it even more difficult to understand how to select the terms. Expressions which create confusion are:

… pequeñas *protuberancias* como *tubérculos* / small tubercle-like bumps – *Ferocactus histrix, Gymnocactus aguirreanus*

… *tubérculos* como pequeñas *protuberancias* espaciadas/ tubercles as small, spaced bumps – *Glandulicactus wrightii*

Returning to the use of the term *tubercle* brings again into discussion the confusion determined by the polysemantic character of this term (*tubercle / warze*).

A first example is found in an old work (1907) by BERGER who uses the term *warze* (equivalent to *tubercle*) with three different meanings: structure of spiny euphorbias, growths on seeds, warts (or other skin growths in humans). A few decades later, *tubercle* [MCCLEARY,

1968] is used in the sense of a formation typical of spiny euphorbias, but also as a sensitive organ in snakes and amphibians.

RIVAS ROSSI (1998) describes *podario* as „protuberancia tuberculada"/tuberculated protuberance, present in genera such as *Epiphyllum*, *Disocactus*, while in globose species (*Melocactus*) *tuberculos* are present. Earlier [RIVAS ROSSI, 1996] defined these terms in a glossary in a way that excluded the relation of synonymy:

Podario, en las cactaceas, protuberancia que queda en el tallo, remanente de la base de las hojas,

Tuberculo, tallo modificado en un organo de reserva, que generalmente esta bajo tierra.

A singular / isolated situation is found by REPPENHAGEN (1984) who in the Latin diagnosis of a new species of *Mammillaria* (*M. hubertmulleri*) uses the term *verrucae* (Latin) as a synonym/homologue for *Warzen* (Germ.), with the meaning of a protuberance bearing thorns.

Despite the recommendation made by the POC after 2011, enough papers appear in which the controversial term appears in the description of taxa [BARTHLOTT & HUNT, 2013; HOUSE & al. 2013; HOLMES, 2016; NAIDU, 2017; PRAT & FRANCK, 2017; ROLIM, 2017; SAROJINIDEVI & VENKATARAJU, 2017; ENDRESS & al. 2018; ROSAS-REINHOLD & al. 2021].

It seems obvious that the recognition or non-recognition of the usefulness of the term *podarium* depends on each individual author. Thus, in the vast work coordinated by Kubitzki, the term *podaria* is used in the description of the family Cactaceae [BARTHLOTT & HUNT, 2013] and Apocynaceae – Subtribe Stapeliinae [ENDRESS & al. 2018], but not in that of the family Euphorbiaceae [WEBSTER, 2014]. SCHRÖDER (2018) prefers the term *podaria* with the meaning "mit der Sprossachse verschmolzene Blattbasen" / leaf bases fused with the stem axis to characterize the species *Ceropegia simoneae*, and with the term *Warzen* / *tubercle* he designates the formations that are present in a specimen named as Form "green bizarre".

It should be noted that the works in Spanish in which the term *podario*(s) appears are relatively numerous [de OLIVEIRA N., 2014; SCHEINVAR & al. 2015; TAPIA & al. 2017; VÁZQUEZ-SÁNCHEZ & al. 2017; AGUIRRE, 2018; ARANDA-PINEDA & al. 2019; ARIAS & AQUINO, 2019; BARRIOS & al. 2019; CAMPOS DÍAZ & al. 2020]. We cannot help but wonder if it is of any significance that the term *tuberculos* is most commonly associated with thickened subterranean formations of some edible plants. GALLEGOS (2014) in the Spanish diagnosis uses the term *podarios*, which in the short English commentary is translated as *tubercles*.

The fact that CASTILLON and RAJAOVELONA in the French paper of 2019 prefer to describe a new species of *Euphorbia* using the term *protubérances* (*protuberances*) is further evidence that there is no unanimously accepted term to designate a unanimously recognized formation.

Terminology aspects

The term *podarium* and its various forms of declension (*podaria* – English, *podariis* – Latin) appear frequently in diagnoses, of course predominantly in Latin. If in the case of cacti it corresponds to tubercles (*mammilla, tuberculos, tubercule*), in the case of the genus *Euphorbia* it most often corresponds to spine-shield [BRUCE & al. 1951; DYER & al. 1958; RAYNAL 1967; CARTER & al. 1981; MALPURE & al. 2016], horny margin, but sometimes also to teeth of the branches ("dentibus") [CROIZAT, 1938b].

It seems that the history of the evolution of this term is little known. The renowned cacti expert RAUH (1979a) notes with amazement that "sukkulente Basalabschnitt der Blätter

wird von Berger (1926)! auch als *Podarium* bezeichnet / succulent basal section of the leaves is described by Berger (1926)! also known as a *podarium*." It is difficult to interpret the meaning of Rauh's surprise, but it seems hard to believe that he did not know that Berger was analyzing a term that was already 70 years old. In the same context, the designation of the term *podarium* as "unique structure (!) for the Cactaceae family" [KOROTKOVA, 2011] or "exclusive character of the Cactaceae family" [TOVAR ROMERO, 2005] is included.

Hence the need to understand the meaning of the term, to use it with as much accuracy as possible, but above all to define it unambiguously. From this perspective, consulting dictionaries or glossaries is not very useful.

As early as 1977, CARTER pointed out that up to the date the term *podarium* had most likely not been included in a Latin dictionary [STEARN, 1966] or a glossary of botanical terms, and he hoped that this would be remedied.

A German dictionary of biology [EICHHORN, 1998] is among the first works in the field containing the definition of the term:

"*Podarium* n *podarium*: mit Sproß verschmolzene Blattbasis bei Cactaceae, eine Struktur bei sukkulenten Euphorbiaarten" / leaf base fused with stem in Cactaceae, a structure in succulent species of Euphorbia.

In 2000, HICKEY & KING set out to produce a glossary "...that includes all the terms most commonly used in describing vascular plants, as well as some that are found in more specialized works." The term *podarium* is missing from their work. The same situation is in the case of the dictionary edited by ALLABY (2006) which was presented as "the most comprehensive and up-to-date dictionary of botany".

Only in 2010, the terms *podarium* and tubercle appear in a glossary published under the auspices of RBG Kew [BEENTJE, 2010], but not as synonymous:

"*podarium*, (in cacti or other succulents) a modified leaf base functioning as the photosynthesising organ;

tubercle, 3. (in ball – or barrel-shaped cacti), cone-shaped protuberances that are enlarged modified leaf bases fused with adjacent stem tissue".

Other definitions present in works dedicated to the Cactaceae family are:

podarium. Collective term for stem-surface protuberances in cacti (pl., *podaria*) [POWELL & WEEDIN, 2004].

podaria: are the swellings often subtending areoles that represent the points of attachment of leaves or bracts that have been lost, or almost lost, in the course of evolution of the highly succulent habit [TAYLOR & ZAPPI, 2004].

podario: "Como podio, pie, sustentáculo; es decir, el internodio que sirve de pie a una rama axilar que brota de su nudo apical. En las cactáceas dícese de la base foliar, dilatada y expendida en forma de un pequeño tubérculo, que sirve de pie a la aréola vegetative" (engl. that is, the internodium that serves as a foot for an axillary branch that sprouts from its apical node. In cacti, it is said to have a leaf base, dilated and expanded in the form of a small tubercle, which serves as a foot for the vegetative areola [VÉLIZ, 2008]; se refere aos espessamentos encontrados no caule, na porção que subtende as aréolas /refers to the thickenings found on the stem, in the portion that subtends the areolas [SOLLER & al. 2014]; base foliar dilatada en forma de pequeño tubérculo, que sustenta a la aréola / leaf base dilated in the form of a small tubercle, which supports the areola [ARIAS & AQUINO, 2019].

According to the specifications in the theories of terminology, Lemaire highlighted a concept for which he proposed an appropriate term to designate it. It is still not clear whether this term is mono or plurisemantic. If it is considered monosemantic, then it should be preferred

in favor of tubercles because "we should not tolerate, in scientific language, a plurality of meanings for one term" [RICKETT, 1954].

Compared to *podarium*, tubercle seems to be an ambiguous term; in Bentjee's work it is mentioned with 3 different meanings:

tubercle, 1. a small tuber, used for any small growth (hypothetically) associated with symbiotic organisms; 2. a small protuberance; 3. (in ball- or barrel-shaped cacti), cone-shaped protuberances that are enlarged modified leaf bases fused with adjacent stem tissue [BEENTJE, 2010].

In addition, in the Oxford Dictionary we also find these two definitions:

1. (anatomy, biology) a small round part, especially on a bone or the surface of an animal or plants;
2. (medical) a small swollen (= larger than normal) area in the lung caused by tuberculosis.

CARTER (1977) said that *scutellum* had too many definitions thus motivating the preference for the term *podarium*; it looks like the term *tubercle* finds itself in a similar situation.

The analysis of the use of the term *podarium* did not aim to exhaust the subject, but to emphasize its lack of clarity. After more than 160 years since Lemaire's proposal, there are still many question marks, but also the possibility that *podarium* will become a forgotten term, as well as "cyrtomê" proposed by the same author.

Conclusion

Changing the meaning of botanical terms with the accumulation of new knowledge is natural, but sometimes they can become obscure. *Podarium* is in such a situation; we cannot say whether it defines a strictly cauline, strictly foliar structure or a concretion of the two, if it designates a unique structure or is actually a collective term. We consider that the term *podarium* sensu Lemaire can be very useful, because it designates a formation specific to succulents and implicitly indicates its heterogeneous, both caulinar and foliar origin.

With the evolution of modern language translation technologies, in the matter of understanding and explaining some terms translations could have a significant contribution to the reduction of linguistic borders or barriers. With the elimination of word-for-word translations and the optimization of finding the right translation, the sources of misunderstandings and errors that are common today will be eliminated.

The translation must observe certain constraints and rules, and faithful observance of the essence of the term in question is a priority. The terminology in question, words without equivalent or misunderstood, can raise real problems. The correct translation is the one adapted to the local language, but also to the exact knowledge of the botanical terminologies, verified and re-verified not by whomever, but by translators with real botanical knowledge, experienced in the targeted field of activity.

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ANATOMICAL INVESTIGATIONS ON *MOMORDICA CHARANTIA* L. PLANTS, NEWLY ACCLIMATED IN ROMANIA

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Abstract: *Momordica charantia* L. (Cucurbitaceae family), medicinal and nutraceutical plant known from Asia, South Africa, South America, the Caribbean region, has been acclimatized in Romania since 1990 until now. This plant, cultivated in the open greenhouses belonging to S.C. HOFIGAL Import Export S.A. has been studied in several projects, among which "Project 160/2014-2017 – MAIA – *Multifunctional and innovative products for safe and bioenhanced functional food from newly cultivated plants in Romania*", coordinated by ICECHIM/ INCDCP Bucharest. Starting from this project, a documentary study was carried out on the *Momordica charantia* plants acclimatized in different countries, and a morpho-anatomical research was initiated on the specimens grown in Romania, in the greenhouses belonging to S.C. HOFIGAL (Voucher BUC 408946-408950). The anatomical observations concerned the organization of the stem, petiole, and leaf lamina, using sets of cross-sectional and paradermal sections, treated with identification substances (IIK) and differential and successive stains (Iodine Green and Carmine Alum). Structural characteristics have been investigated under optical microscopy and documented through original photographic images and a set of dimensional data, data which are only partially found in the specialised literature. The results of our research are generally within the existent anatomical patterns. However, certain particular aspects have been noticed, regarding the epidermal cells, mechanical tissues, conducting tissues and mesophyll, completing the knowledge regarding the anatomy of acclimatized *Momordica charantia* plants.

Key words: Cultivated *Momordica* plants, nutraceutical importance, stem and leaf structure, optical microscopy.

Introduction

Momordica charantia is a tropical and subtropical plant of the *Cucurbitaceae* family, cultivated at large in Asia, Africa, the Caribbean for its edible fruits (bitter cucumber).

The plant is renowned for its many beneficial effects in the fight against diabetes, and its complications (eye diseases, cataract, obesity, hyperglycemia, diabetic foot), for its anticancer / antimutagenic / antitumor, antiviral (among which anti-AIDS), and antibacterial properties. Other important uses are based on its fungicidal effects against phytopathogens and insecticides / larvicides / antipupal effect on plant pests and its beneficial effects against skin conditions, antipsoriasis, anti-wound healing, anti-cardiovascular diseases. The plant also has a hepatoprotective effect, an analgesic effect and is considered a good source of food with a tonic effect on the body [WALTERS & DECKERS, 1988; GROVER & YADAV, 2004; BEHERA & al. 2010, 2011; KUMAR & al. 2010; KUMAR & BHOWMIK, 2010; GUPTA &

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al. 2011; MAHMOOD & al. 2012; AGARWAL, 2014; NAGARANI & al. 2014a, b; SAIFI & al. 2014; ANILAKUMAR & al. 2015; TCHEGHEBE & al. 2016; MAHMOUD & al. 2017; DE OLIVEIRA & al. 2018; RAHMAN & al. 2018; ASNA & al. 2020; KOLE & al. 2020; ŞESAN, 2020].

Over time, the plant has been acclimatized in many countries, and since 1990 it has been cultivated in Romania as well. The history of this culture starts with the first seeds brought from Nepal by biologist PhD. Ovidiu BOJOR, mentioned by biologist PhD. STOIANOV (2002), seeds which were cultivated in the open greenhouses of S.C. HOFIGAL Import Export S.A. At the same time, the first cultures of *Momordica charantia* were established at SCDL (Vegetable Growing Research and Development Station) Buzău, within the ASAS (Academy of Agricultural and Forestry Sciences) by VÎNĂTORU (2015) and by VÎNĂTORU & al. (2019).

The technology for cultivating bitter cucumber was disseminated in Romania through a CD created by the company RENTROP & STRATON (CĂPĂŢÂNĂ), and after the 2000s, through various projects, such as those coordinated by: ONISEI (2016-2017), ŞESAN (2017), ŞESAN & al. (2018), VÎNĂTORU & al. (2019), in the south, or CRIŞAN & HĂLMĂJAN (2007) and KESERU & al. (2016), in the western areas of Romania.

Anatomical studies of the vegetative organs have been carried out internationally on *Momordica charantia* acclimatized plants, grown for nutraceutical purposes [AGUORU & OKOLI, 2012; SONKAR & al. 2014; ERŐZ POYRAZ & DERDOVSKI, 2016; SHETHI & al. 2018; SÁ & al. 2018]. In Romania, anatomical research on the vegetative organs of *Momordica charantia* cultivated plants has been carried out by: SĂVULESCU & HOZA (2010), LAGUNOVSCHI-LUCHIAN & al. (2017), LUCHIAN & (IORDACHE) TEODOSIU (2019). All these studies highlighted the existence of a similar organisation plan of the plant, but also the existence of certain particularities supporting their structural variability.

This paper aims to perform the morpho-structural characterization of the local *Momordica charantia* population, cultivated in open greenhouses in the experimental fields of HOFIGAL, Romania. Our results will complete the existing information on the anatomy of the vegetative organs of the *Momordica charantia* plants acclimatized in different countries, including Romania.

This paper is further aimed at creating a reference of structural data and relevant optical microscopy images, to be used in assessing future *Momordica charantia* plants, cultivated in the greenhouses of HOFIGAL, Romania, in several experimental variants, created with the purpose of enhancing their medicinal values.

Materials and methods

Biological material: stems and leaves harvested from the *Momordica charantia* plants (anthesis stage) cultivated in open greenhouses in the experimental fields of S.C. HOFIGAL Export Import S.A., Romania, during 2015-2016 and preserved in 70% alcohol.

Processing methods. For histo-anatomical evaluation, the usual methods used in plant anatomy were used [ŞERBĂNESCU-JITARIU & al. 1983].

The *Momordica charantia* stems and leaves were cross-sectioned manually: in the median area of the stem internode in the middle of the stem, in the median area of the petiole, in the median area of the central leaf segment (Figure 1). The sections were coloured using two differential stains, applied successively (Iodine Green and Carmine Alum) [ŞERBĂNESCU-JITARIU & al. 1983; SÂRBU & al. 2014; SÂRBU & al. 2018], and IIK was

used as a starch recognition substance. For analysing the characteristics of the epidermis, paradermal sections were performed at the established levels of the stems and leaves.

All microscopic slides were analysed under normal and polarized light, using a DOCUVAL optical microscope. Photomicrographs were taken with a digital camera (NIKON D90).

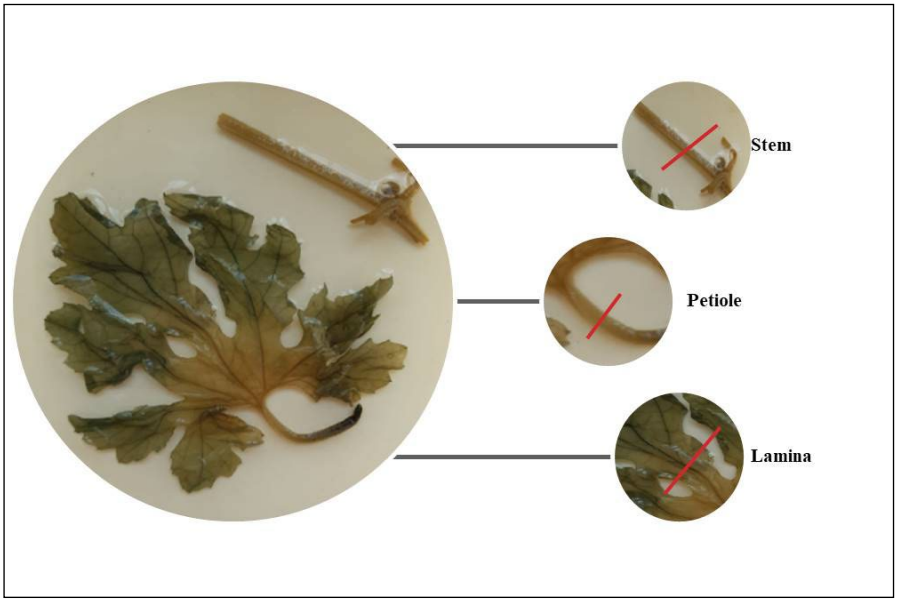

Figure 1. *Momordica charantia* – sectioning levels (cross sections, paradermal sections).

Results

Stem. *Momordica charantia* is an annual herbaceous plant with an ascending stem (2-3 m), pubescent and provided with tendrils.

In cross section the contour of the stem has 5 obvious angles (diameter 3.8/4.0 mm), corresponding to the morphological appearance of the stem, which longitudinally has 5 ridges and 5 valleculas (Figure 2, Table 1). The epidermis is unilayered, composed of small epidermal cells (in cross section: 10-12 µm long; 8-10 µm wide), with the outer tangential wall slightly thickened and covered by a 2.0-2.2 µm thick cuticle. The epidermis differentiates rare stomata, long uniseriate multicellular non-glandular trichomes (15-20 cells) and short glandular trichomes, which have a single secretory cell apically (Figure 3, Figure 4). Calcium carbonate crystals (double cystoliths) are present in the epidermal cells (Figure 5).

Below the epidermis, a cortical angular collenchyma is present in the area of the ridges, where their apical area includes 8-10 layers of cells and reaches a thickness of 120-150 µm (Figure 6, Table 1). Otherwise, the cortex is parenchymal, with slight spaces between the cells, composed of 5-6 layers of cells with slightly thickened walls, cells in which starch deposits have been identified (recognition reaction with IIK) (Figure 7).

The central cylinder follows the shape of the stem, also being 5-sided (Figure 2) and slightly oval (2.0 mm length / 2.3 mm width) (Table1). Its outline is marked by a thick (7-10 layers of cells) and continuous sclerenchyma area (Figure 2, Figure 3, Figure 7), in a

pericyclic position. The fundamental parenchyma of the central cylinder contains 10 bicollateral vascular bundles, arranged in two rings: 5 large, central bundles and 5 smaller, peripheral bundles (Table 1). Each bundle has an external phloem area, an intrafascicular vascular meristematic tissue (procambium), a xylem area, and an internal phloem area (Figure 8). The metaxylem has an exarch position, and the protoxylem has an endarch position. In the central cylinder, the xylem is well represented and includes 15 large wooden vessels (diameter ≥ 100 µm) (Table 1). The medullary rays and the medullary parenchyma are parenchymal.

Table 1. Analysed *Momordica charantia*, stem parameters (cross section through the internode).

Parameters	**Measurements/size**
Stem diameter	3.8 mm/4.0 mm
Epidermal cell	10.0-12.0 µm length 8.0-10.0 µm width
$CaCO_3$ crystals (cystoliths)	15-30 µm diameter
Cuticle	2.0-2.2 µm thickness
Sclerenchyma	100.0-150.0 µm thickness
Angular collenchyma from the apical area of the ribs	120.0-150.0 µm thickness
Diameter of the central cylinder	2.0 mm/2.3 mm
Conducting bundles/central cylinder	10.0 (5.0 large central bundles, 5.0 small bundles of ribs)
Xylem vessels (≥ 100 µm diameter)/central cylinder	15.0
Large central conducting bundles	700.0-800.0 µm length 500.0-600.0 µm width
Small conducting bundles of ribs	400.0-500.0 µm length 350.0-400.0 µm width

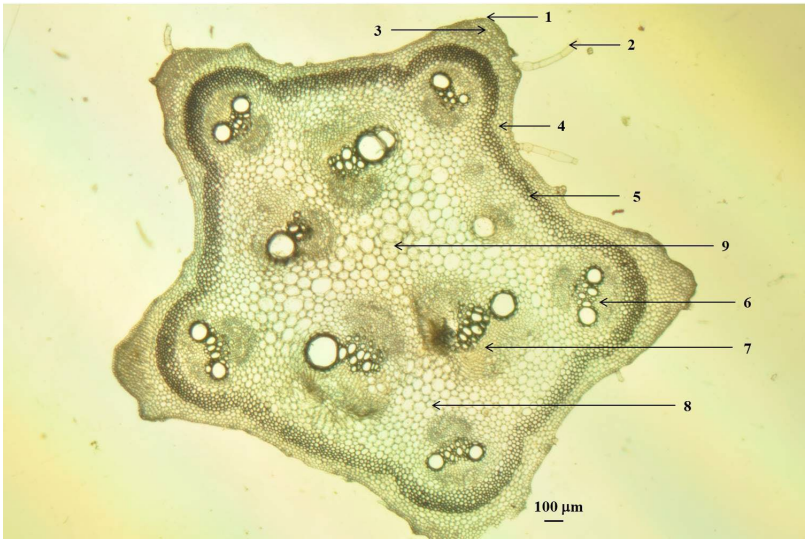

Figure 2. *Momordica charantia*, cross section through the stem internode (colorants: Iodine Green and Carmine Alum): 1 – epidermis, 2 – tector trichome, 3 – collenchyma, 4 – parenchymal cortex, 5 – sclerenchyma, 6 – rib conducting bundle, 7 – central conducting bundle, 8 – parenchymal rays, 9 – parenchymatic pith.

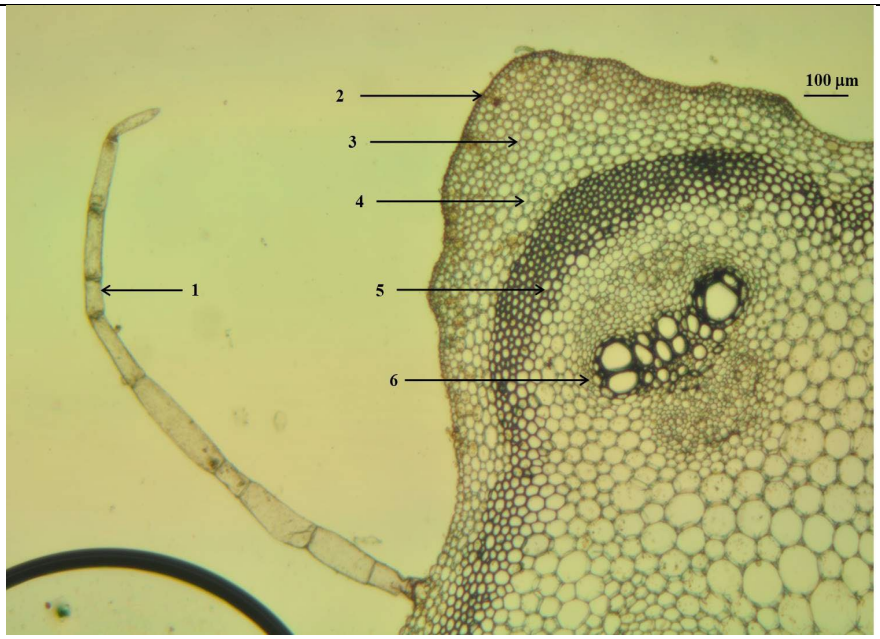

Figure 3. *Momordica charantia*, cross section through the stem internode (colorants: Iodine Green and Carmine Alum): 1 – multicellular tector hair, 2 – epidermis, 3 – angular collenchyma, 4 – parenchymal cortex, 5 – sclerenchyma, 6 – bicollateral vascular bundle from the ridge.

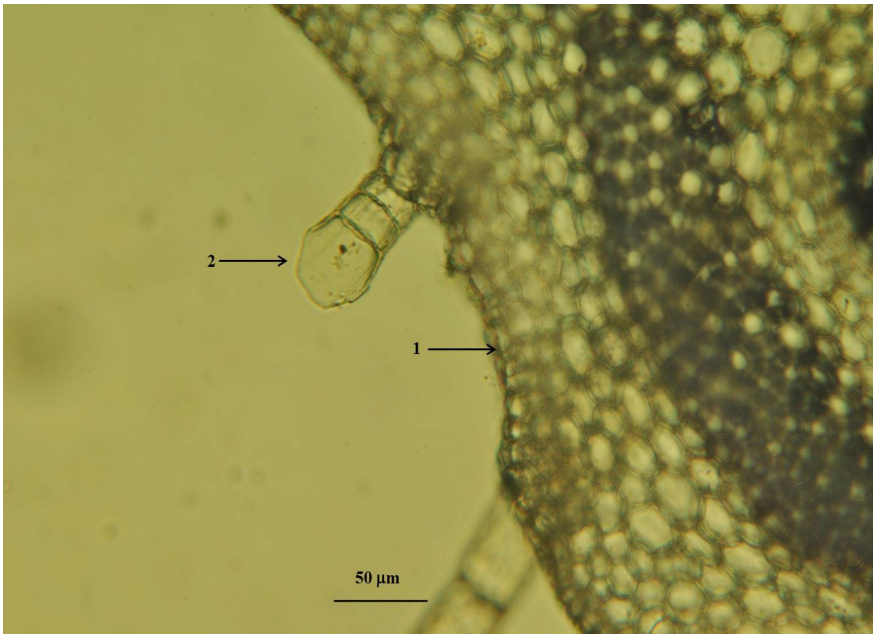

Figure 4. *Momordica charantia*, cross section through the stem internode (colorants: Iodine Green and Carmine Alum): 1 – epidermis, 2 – multicellular glandular hair.

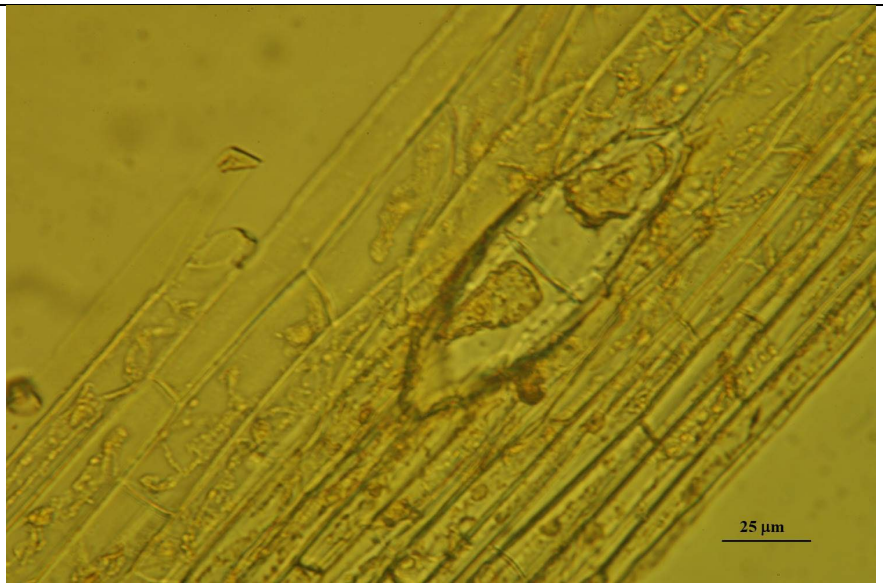

Figure 5. *Momordica charantia*, paradermal section at the level of the stem internode: the epidermis and a double cystolith are observed.

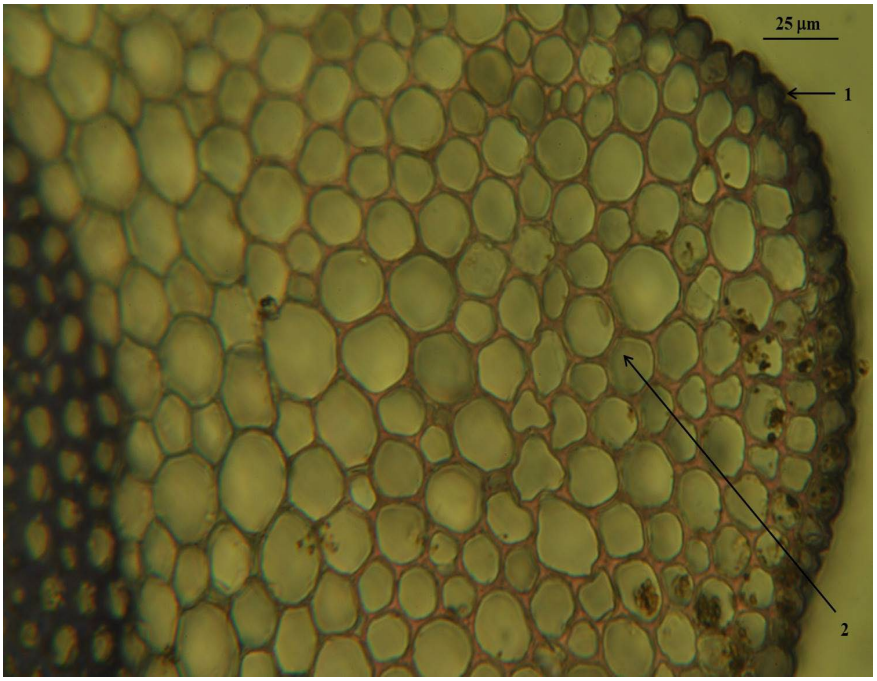

Figure 6. *Momordica charantia*, cross section through the stem internode (colorants: Iodine Green and Carmine Alum): 1 – epidermis, 2 – angular collenchyma from the apical area of the ribs.

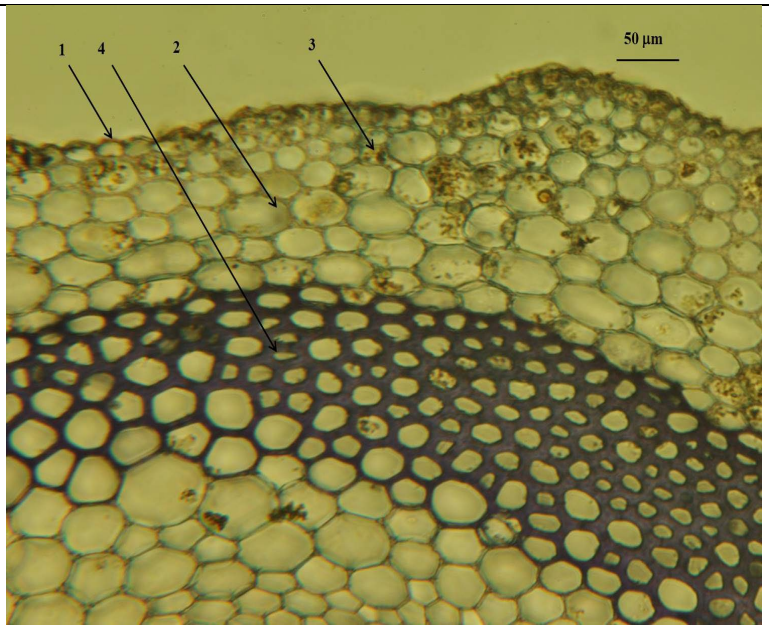

Figure 7. *Momordica charantia*, cross section through the stem internode (colorants: Iodine Green, Carmine Alum, IIK): 1 – epidermis, 2 – cortex, 3 – starch granules, 4 – sclerenchyma.

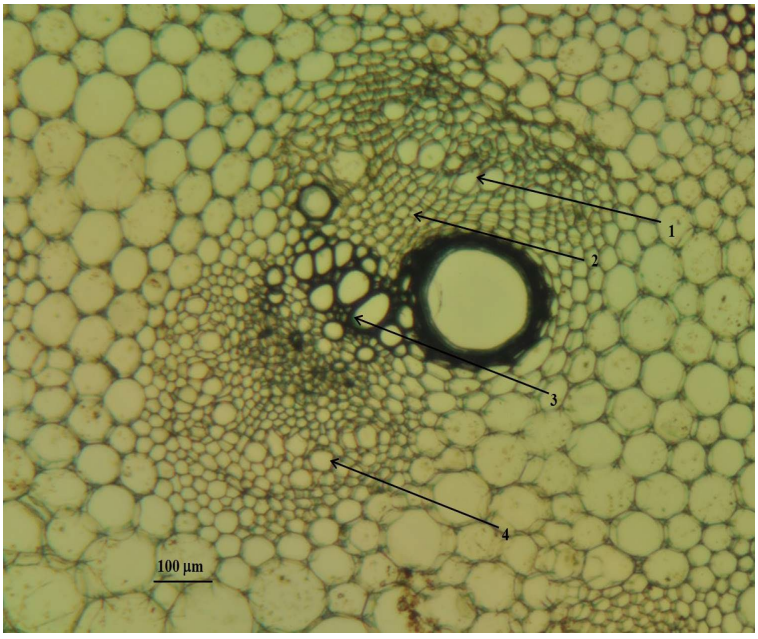

Figure 8. *Momordica charantia*, cross section through the stem internode with the highlighting of a bicollateral vascular bundle (colorants: Iodine Green and Carmine Alum): 1 – outer phloem, 2 – fascicular procambium, 3 – xylem, 4 – inner phloem.

Petiole. The leaves are alternate, simple, with membranous lamina, palmatilobate, with 5-7 lobes (Figure 1). The petiole and the veins of the lamina are pubescent.

In cross section through its median area, the petiole shows a slightly angled circular outline, modified only by the presence of a shallow adaxial groove, and of the two appendages enclosing it (Figure 9). The diameter of the petiole varies between 2.5 mm - 2.7 mm (Table 2), and its structure is monosymmetric. From a histological point of view, at least four types of tissues are present and characteristic: protective, mechanical, parenchymal, and conducting.

The epidermis is unilayered. The epidermal cells are large, relatively isodiametric cells (16-18 µm long and 13-15 µm wide) (Table 2) and are covered by a cuticle (2.2-2.5 µm thickness), which presents cuticular ridges, more numerous and obvious in the area of the adaxial groove and of the two appendages (Figure 10). The epidermis differentiates rare stomata, uniseriate multicellular non-glandular trichomes and rare long multicellular stalked glandular trichomes (feet composed of 6-7 cells), with multicellular glandular head. Calcium carbonate crystals (cystoliths) are present in the epidermal cells.

Under the epidermis there is a continuous ring of angular collenchyma, with variable thickness on the circumference of the petiole (2-5 layers of cells) and better developed at the level of the adaxial groove and appendages (Figure 11).

The conducting tissues are organized into 11 bundles, of which 7 are located in the meatic fundamental parenchyma and 4 in the two appendages. Five conducting bundles in the fundamental parenchyma are obviously larger and of the bicollateral type (Table 2, Figure 11). In these conducting bundles the xylem is well represented (Figure 11, Figure 12), and at the periphery of the external phloem there are sheaths of amyliferous cells (IIK) (Figure 12).

Table 2. Analysed *Momordica charantia*, petiole parametrs (cross section in the median zone).

Parameters	**Measurements/size**
Petiole diameter	2.5-2.7 mm
Epidermal cells	16.0-18.0 µm length 13.0-15.0 µm width
$CaCO_3$ crystals (cystoliths)	20.0-30.0 µm diameter
Cuticle	2.2-2.5 µm thickness
Conducting bundles	11.0
Large conducting bundles from the fundamental parenchyma	5 (400.0-600.0 µm length, 200.0-300.0 µm width)

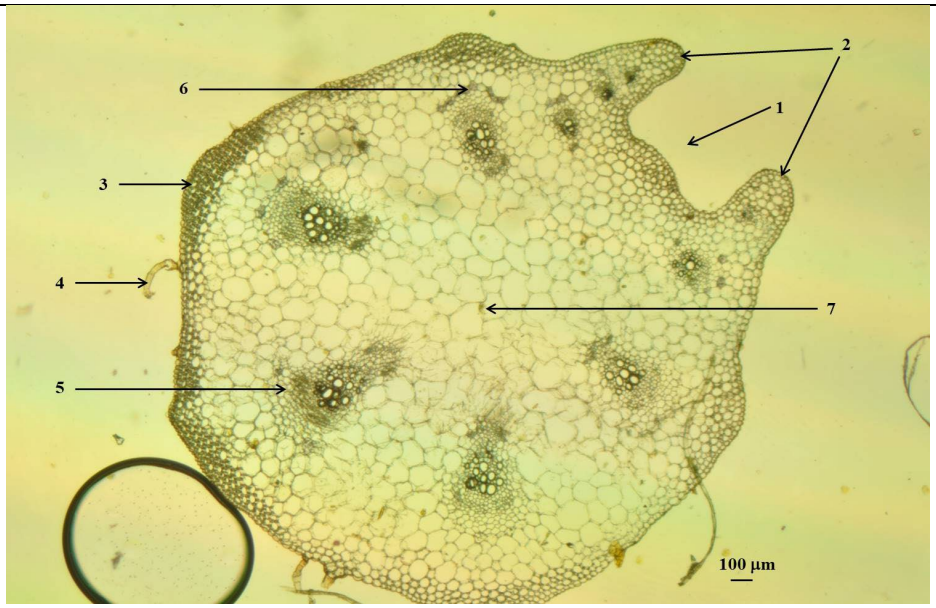

Figure 9. *Momordica charantia*, cross section through the petiole (colorants: Iodine Green and Carmine Alum, IIK): 1 – adaxial groove, 2 – appendages, 3 –angular collenchyma, 4 – multicellular tector trichome, 5 – large bicollateral vascular bundle, 6 – starch cells, 7 – ground parenchyma.

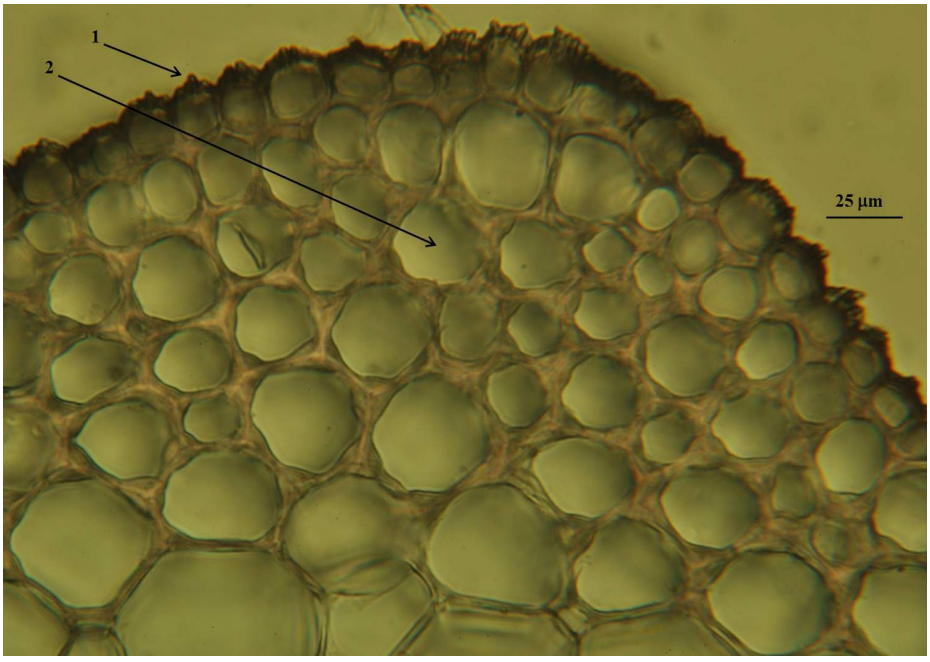

Figure 10. *Momordica charantia*, cross section through the petiole (colorants: Iodine Green and Carmine Alum): 1 – epidermal cells covered by a ridged cuticle, 2 – angular collenchyma (detail).

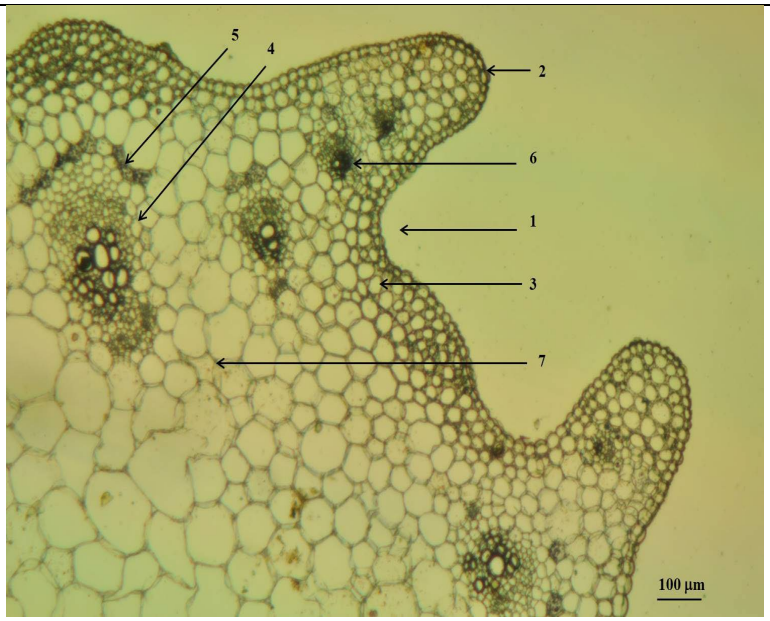

Figure 11. *Momordica charantia*: cross section through the petiole (colorants: Iodine Green and Carmine Alum, IIK): 1 – adaxial groove, 2 – appendage, 3 –angular collenchyma, 4 – large bicollateral vascular bundle, 5 – starch cells, 6 – small vascular bundle, 7 – ground parenchyma.

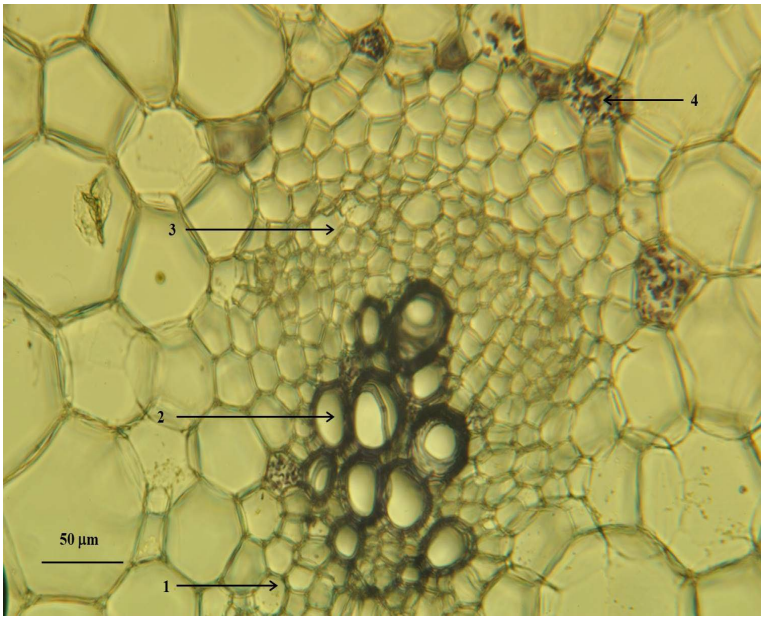

Figure 12. *Momordica charantia*: cross section through the petiole, detail of a bicollateral vascular bundle (colorants: Iodine Green and Carmine Alum, IIK): 1 – inner phloem, 2 – xylem, 3 – outer phloem, 4 – starch cells.

Lamina. The epidermis seen from above highlights the presence of the epidermal cells, arranged in a single layer, of anomocytic stomata, of uniseriate multicellular non-glandular trichomes with walls impregnated with $CaCO_3$ and of multicellular stalked glandular trichomes, with multicellular glandular head. The lamina is amphistomatic (stomatal index: upper epidermis = 44.79 stomata/mm^2, lower epidermis = 146.19 stomata/mm^2).

Both epidermal layers have heterodiametric epidermal cells, with obviously wavy walls. The cells of the lower epidermis are slightly larger (Figure 13, Figure 14, Table 3). Some of the cells of the lower epidermis have cystoliths (50 µm / 40 µm), arranged in groups of 2-3-4 (Figure 15).

In cross section, the shape of the median nervure of the leaf segment is biconvex (1000 µm / 900 µm), with both sides prominent: the adaxial side is convex and the abaxial side is obviously convex (Figure 16).

Both epidermis of the nervure show cells that are approximately isodiametric, all walls thin. Multicellular glandular trichomes with multicellular glandular head and uniseriate multicellular non-glandular trichomes of up to 1200 µm in length (Figure 17, Figure 18, Figure 19) are present. The lower epidermis is covered by a cuticle with cuticular ridges (Figure 20).

Adaxially, subepidermally, 4-5 layers of angular collenchyma are identified, and abaxially, above the lower epidermis, a continuous ring of angular collenchyma consisting of 2-3 layers of mechanical cells (Figure 16) is identified.

In the centre of the nervure there is a single bicollateral conducting bundle (300-350 µm diameter), located in the meatic fundamental parenchyma. The external phloem cordon is small, and the internal one is voluminous (Figure 21).

The mesophyll shows a dorsiventral organization (Figure 22): one layer of palisade cells (40-50 µm thickness) and an area of spongy tissue, composed of a layer of heterodiametric collector cells and 5-6 layers of relatively isodiametric cells, with small intercellular spaces between them. The layer of collector cells is better represented in the area where the cells of the lower epidermis contain large cystoliths (40-50 µm diameter). The spongy parenchyma form ~ 50% of the thickness of the mesophyll and its cells show accumulations of starch. In the mesophyll there are secondary ribs containing collateral conducting bundles, composed of few conducting elements.

Table 3. Analysed *Momordica charantia*, lamina parameters (cross and paradermal sections).

Parameters	Measurements/size
Median nervure (cross section)	1000.0 µm length 900.0 µm width
Conducting bundles (cross section)	300.0-350.0 µm diameter
Lamina (cross section)	180.0-190.0 µm thickness
Cuticle (cross section)	2.0-2.5 µm thickness
Cells of the upper epidermis (paradermal section)	25.0 µm width 50.0 µm length
Cells of the lower epidermis (paradermal section)	30.0 µm width 60.0 µm length
Palisade tissue (cross section) – 1 layer	40.0-50.0 µm thickness
Palisade cells (cross section)	40.0-50.0 µm length 25.0 µm width
Spongy mesophyll (cross section)	90.0-100.0 µm thickness
$CaCO_3$ crystals (cystoliths)	50.0 µm/ 40.0 µm diameter
Stomatal index	upper epidermis = 44.79/mm^2 lower epidermis = 146.19/mm^2

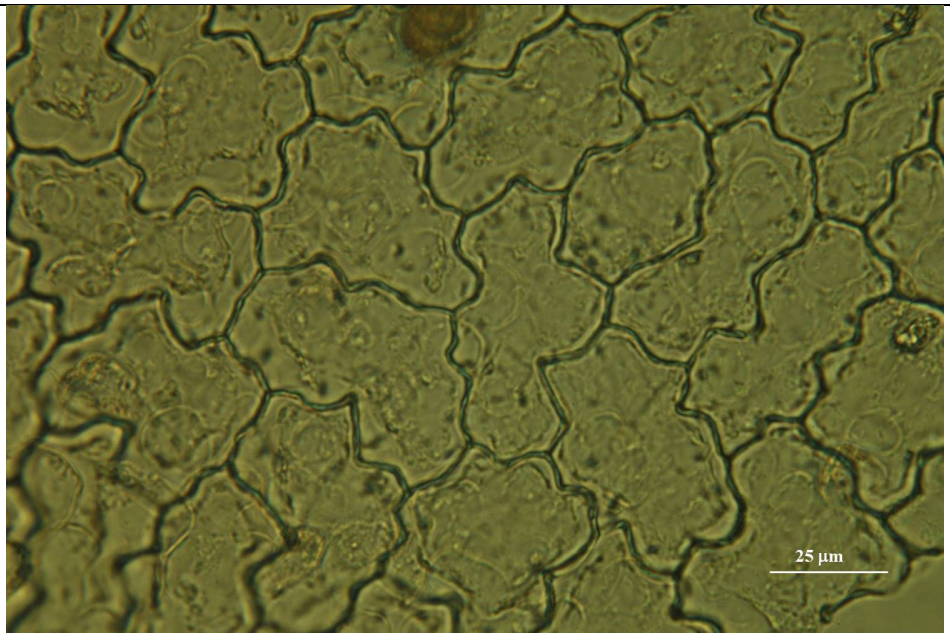

Figure 13. *Momordica charantia*, paradermal section at the lamina level: upper epidermis of the lamina in apical view.

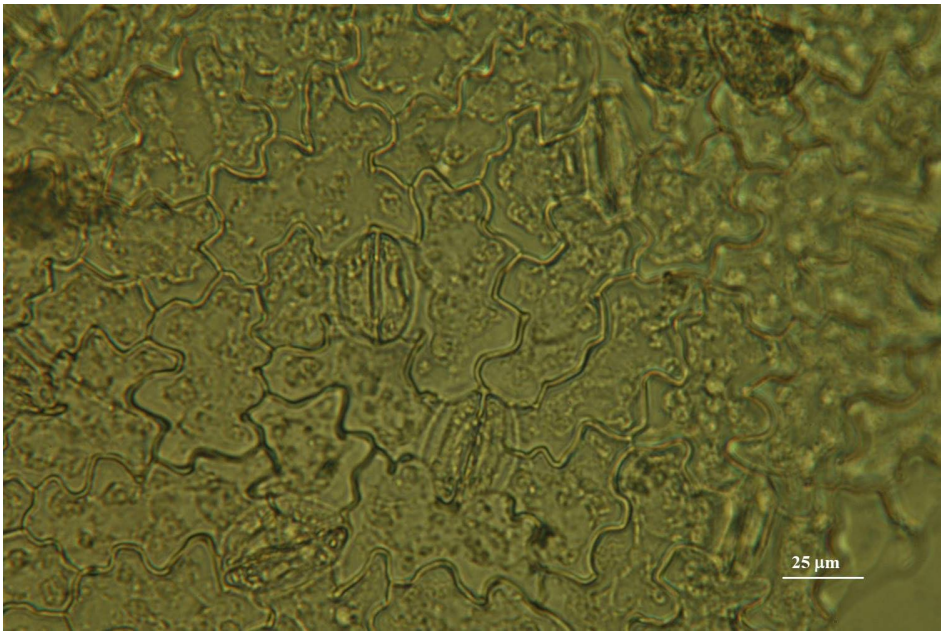

Figure 14. *Momordica charantia*, paradermal section at the lamina level: lower epidermis in apical view.

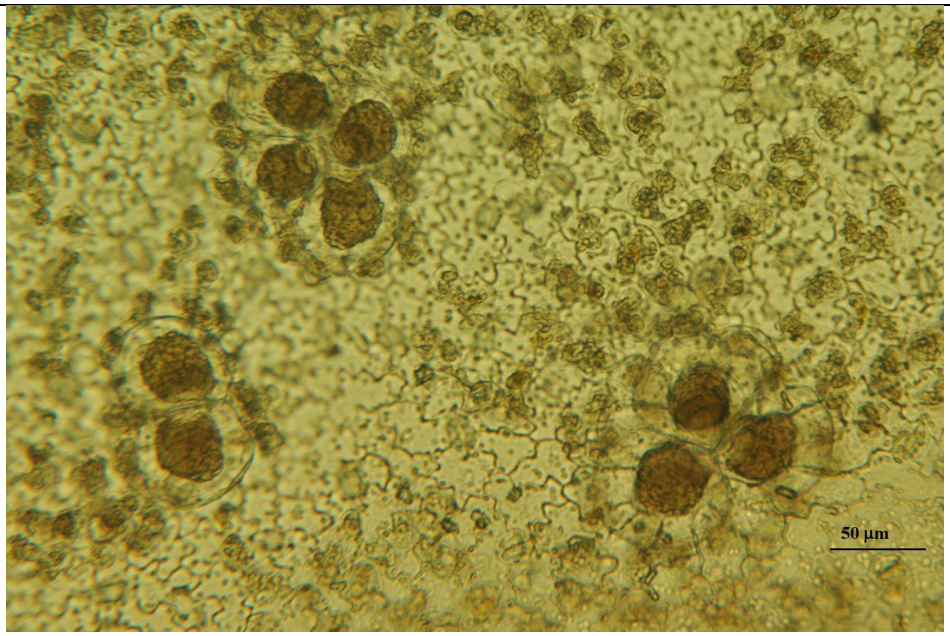

Figure 15. *Momordica charantia*, lower epidermis in apical view:
cystoliths arranged in groups of two, three and four respectively are highlighted.

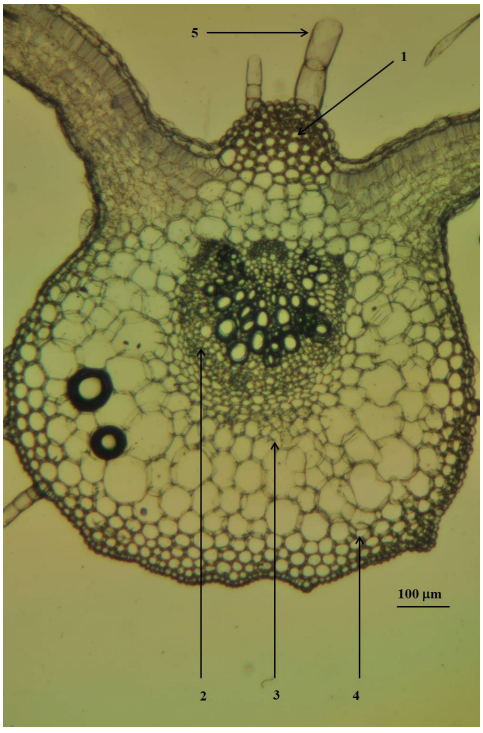

Figure 16. *Momordica charantia*, cross section through the lamina of the leaf segment, at the level of the median ribs (colorants: Iodine Green and Carmine Alum): 1 – adaxial collenchyma, 2 – bicollateral vascular bundles, 3 – parenchyma, 4 – abaxial collenchyma.

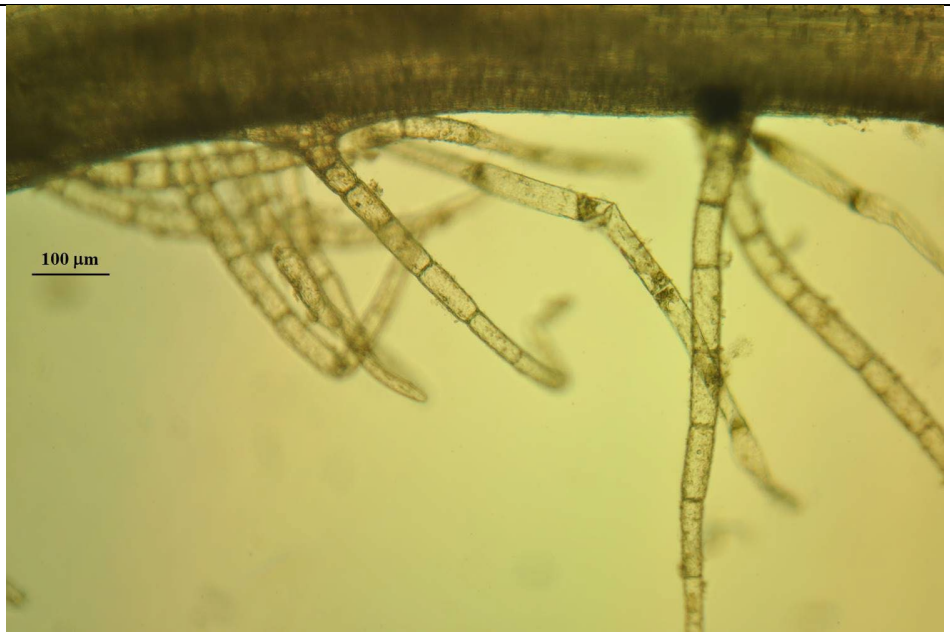

Figure 17. *Momordica charantia*, lower epidermis of the median rib of the leaf segment: multicellular tector hairs.

Figure 18. *Momordica charantia*, multicellular tector hair.

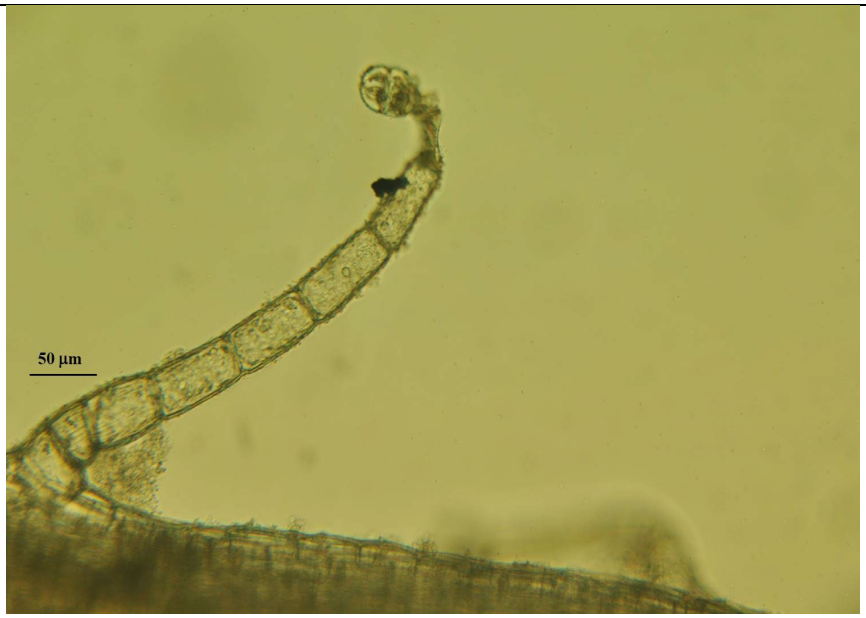

Figure 19. *Momordica charantia*, lower epidermis of the median rib of the leaf segment: multicellular secretory hair.

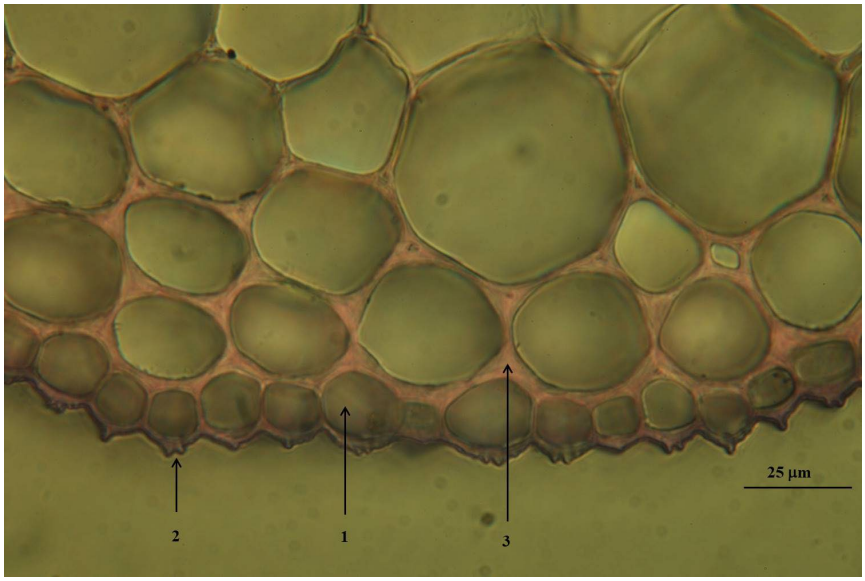

Figure 20. *Momordica charantia*, cross section through the leaf with highlighting of the abaxial face of the median rib (colorants: Iodine Green and Carmine Alum): 1 – epidermal cells, 2 – cuticle with cuticular ridges, 3 – angular collenchyma.

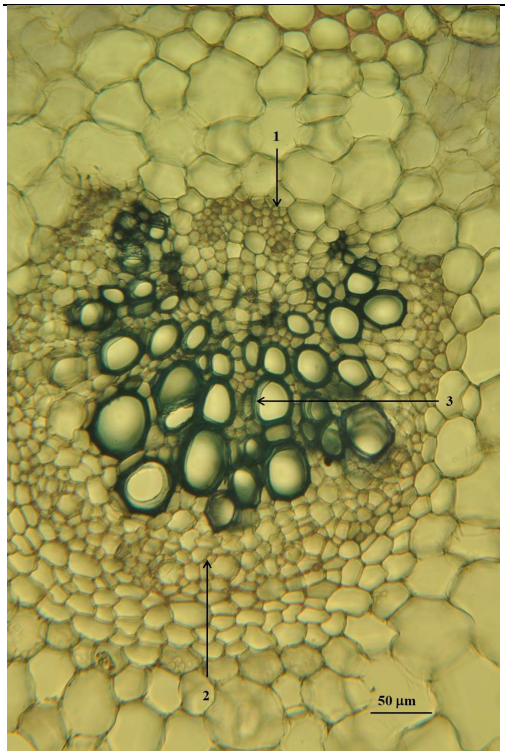

Figure 21. *Momordica charantia*, cross section through the leaf segment at the level of the median rib, with highlighting the bicollateral vascular bundle (colorants: Iodine Green and Carmine Alum): 1 – outer phloem, 2 – inner phloem, 3 – xylem.

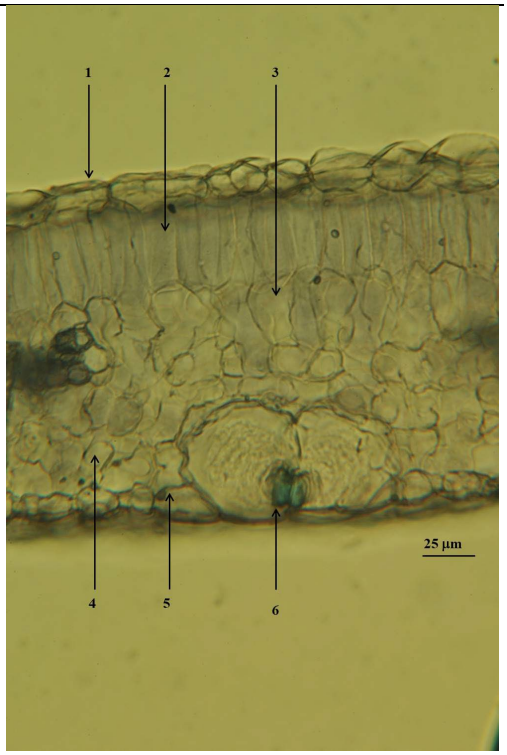

Figure 22. *Momordica charantia*, cross section through the lamina (colorants: Iodine Green and Carmine Alum): 1 – upper epidermis, 2 – palisade tissue, 3 – collecting cells, 4 – spongy tissue, 5 – lower epidermis, 6 – double cystolith.

Discussions

This study provides detailed data regarding the anatomical characteristics of the *Momordica charantia* plants acclimatized in the Hofigal greenhouses, Romania.

The structure of the analysed above-ground vegetative organs corresponds to the descriptions existing in the literature regarding the *Momordica charantia* plants acclimatized in other countries (Bangladesh, Brazil, Nigeria, Turkey etc.), but there is also a series of particular aspects that we point out, aspects related to epidermal cells, mechanical tissues, conducting tissues and mesophyll tissues.

Epidermal cells

Regarding the epidermal cells, our data, supported by the optical microscopy images, show that the cuticle of the epidermal cells of the petiole and of the lamina ribs is thick and shows obvious cuticular ridges. In the specialized literature [SĂVULESCU & HOZA, 2010; SHETHI & al. 2018; ERŐZ POYRAZ & DERDOVSKI, 2016; AGOURU & OKALI, 2012;

SÁ & al. 2018] it is mentioned that the acclimatized *Momordica charantia* plants present a thin and smooth cuticle.

A further aspect refers to the presence of $CaCO_3$ crystals (cystoliths) in epidermal cells. In the case of the plants analysed in the present study, the presence of cystoliths was identified both in the epidermis of the stem and petiole, and especially in the lower epidermis of the lamina. Publications on this topic show that there are variations regarding this characteristic: cystoliths have not been identified [SĂVULESCU & HOZA, 2010], have been identified in the epidermis of the lamina [GILL & KARATELA, 1982; ERŐZ POYRAZ & DERDOVSKI, 2016; SÁ & al. 2018; SHETHI & al. 2018], and less often in the epidermis of the stem [ERŐZ POYRAZ & DERDOVSKI, 2016]. With regard to the epidermis of the petiole, only the presence of raphides [SHETHI & al. 2018] or druses [SÁ & al. 2018] was signalled.

Mechanical tissues

Mechanical tissues are well represented in the structure of *Momordica charantia* plants analysed in this study, both in the stem (collenchyma 8-10 layers of cells, sclerenchyma 7-10 layers of cells) and in the petiole (collenchyma 3-5 layers of cells). This aspect could also influence, to a certain extent, the vigour of the shape of these plants. For plants acclimatized in other countries, some variations of this characteristic have been reported. In the plants grown in Nigeria [AGOURU & OKALI, 2012], for example, the stem has a collenchyma composed of 4-5 layers of cells and a sclerenchyma composed of 6 layers of cells, while the petiole presents a two-layered collenchyma. The plants cultivated in Brazil [SÁ & al. 2018] have 1-3 layers of collenchyma cells, in the structure of the petiole.

Conducting tissues

Momordica charantia, being part of the *Cucurbitaceae* family, is characterized by the presence of bicollateral conducting bundles. The literature emphasizes this aspect and points out that there are differences regarding the number of conducting bundles in the organs of different acclimatized *Momordica charantia* plants. Our data shows the existence of 10 conducting bundles of different dimensions (5 central and 5 peripheral bundles) in the stem, which was also reported for other cultivated plants [SĂVULESCU & HOZA, 2010; ERŐZ POYRAZ & DERDOVSKI, 2016; SÁ & al. 2018]. However, the plants grown in Bangladesh [SHETHI & al. 2018] only have 8 conducting bundles, 3 central and 5 peripheral bundles.

Regarding the petiole, the numerical variation of the conducting bundles was also observed. Our research has highlighted the presence of 11 conducting bundles, values also confirmed by SĂVULESCU & al. (2010). The research performed by AGOURU & OKALI (2012) mentions 10 conducting bundles, while the data obtained by SHETHI & al. (2018) and SÁ & al. (2018) mention only 7 conducting bundles.

Numerical variations of the conducting bundles were also reported for the median nervure of the leaf segments. Our results, but also certain data from the literature [SĂVULESCU & HOZA, 2010; ERŐZ POYRAZ & DERDOVSKI, 2016; SÁ & al. 2018], attest the presence of a single, large, bicollateral conducting bundle in the nervure. However, for the plants in Bangladesh [SHETHI & al. 2018], three conducting bundles were identified, a large one and two small ones.

Mesophyll tissues

Regarding the mesophyll of the acclimatized *Momordica charantia* plants, some structural variations can also be specified. Our data certify the presence of unilayered palisade tissue in the lamina, similarly with what was reported by SÁ & al. (2018) and SHETHI & al. (2018). The research conducted by SĂVULESCU & HOZA (2010) and ERŐZ POYRAZ & DERDOVSKI (2016) supports both the existence of a uni-bilayered palisade tissue in the structure of the mesophyll. Our observations also highlight the presence in the mesophyll structure of a layer of collector cells. It is located under the palisade tissue of the lamina and is better developed in the area of the mesophyll, where cystoliths form in the cells of the lower epidermis.

Conclusions

Momordica charantia (*Cucurbitaceae*) is a nutraceutical plant cultivated in many countries around the world, due to its recognized medicinal properties.

The anatomical aspects investigated in the *Momordica charantia* plants cultivated in Romania (the Hofigal greenhouses), have allowed for the characterization of these plants, compared to the plants grown in other countries. Thus, it was noticed that the general organization of the studied vegetative organs is similar, but there are also certain particularities: thick cuticle provided with cuticular ridges, better represented mechanical tissues, the presence of isolated or grouped amyliferous cells, the presence of collector cells located in the mesophyll, the presence of cystoliths of different shapes, sizes, and placements.

In this sense, the following structural aspects are to be highlighted:

- the central cylinder of the stem is an eustel, with 10 bicollateral conducting bundles, disposed as two concentric rings;
- the mechanical tissues are better represented, compared to other *Momordica charantia* plants cultivated; the stem contains collenchyma and sclerenchyma, the leaf only differentiates collenchyma;
- the petiole presents an adaxial groove and two appendages; it has a monosymmetric structure, with separate vascular bundles (bicollateral); in exarh position perifascicular amyliferous cells have been signalled;
- the epidermis of the petiole and of the median nervure of the leaf lobes is covered by a thick cuticle, which forms obvious cuticular ridges;
- the epidermis of the stem, petiole and median nervure of the leaf segments differentiates: long uniseriate multicellular non-glandular trichomes with the wall impregnated with $CaCO_3$, short glandular trichomes with unicellular gland, long glandular trichomes with multicellular gland;
- the plant forms $CaCO_3$ crystals (cystoliths): solitary or in groups of two in the epidermis of the stem and petiole and in groups of 2-3-4 in the lower epidermis of the lamina;
- the lamina is amphistomatic, with anomocytic stomata, with 70% more numerous abaxially;
- the palisade tissue is unilayered; the presence of a layer of collector cells, was observed.

In this study, dimensional assessments have been performed for the structures of the analysed organs; these data provide additional information, than are found only sporadically in the literature.

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INVESTIGATING EARLY SIGNS OF ENDOREDUPLICATION IN *ARABIDOPSIS* SHOOT: AN UNKNOWN FACT

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Abstract: Endoreduplication is alternative form of cell cycle that involves the replication of DNA without mitosis. It commonly occurs in various tissues of plants like pavement cells of leaf, trichomes and sepals of flower and led to increase in size of the cell. Shoot apical meristem (SAM) is a region from where the aboveground organs of the plant arise. The cells in the meristem remain in meristematic state (mitotic) and get displaced from there to form the differentiated tissues. But how the nuclear DNA synthesis vary from the meristematic cells to the differentiated tissues is not very well studied. It has been observed through the present study that the cells in shoot tip of *Arabidopsis* are diploid in nature and starts to endoreduplicate at 1cm away from the shoot, down the stem which was justified by the flow cytometer-based DNA analysis of different *Arabidopsis* tissues. Petal is known to be the most variable part of the flower with different color, shape, size and fragrance but little is known about its characterization. The cell identities in petal are diverse with different cell types. One is small sized distal cells and the other is large sized proximal cells. The present study has addressed that the large size of proximal cells of petal is not merely a cell expansion but is something related to endoreduplication. This was evidenced by the DNA estimation of FACS (Florescent activated cell sorter) sorted petal cells of *Arabidopsis*. This is the first study in which SAM, stem, leaf and petal cells are taken into account for ploidy analysis by flow cytometry. In shoot apex we did not see endoreduplication however as the cells descend towards the stem or leaf they enter into differentiation pathways and undergo endoreduplication. On the other hand, different ploidies in petal cells shows the signs of endoreduplication which can be a way towards differentiation.

Keywords: ploidy, endoreduplication, petal, florescent activated cell sorter, *Arabidopsis.*

Introduction

In plants, organs are formed during embryogenesis and continuous the development through the activity of specialized tissue called shoot and root meristem. The cells in the meristem remain in the proliferating state and maintains a balance between the cell proliferation and differentiation throughout the development. In response to various signals, the cells displaced from meristem and form differentiated tissues (stem or lateral organs) [STEEVES & SUSSEX, 1989]. Most of the differentiated tissues have a tendency to undergo endoreduplication which is an alternate form of cell cycle [BARLOW & al. 1978]. During this phenomenon, nuclear DNA is replicated without mitosis due to which amount of DNA becomes greater than 2C. The indirect effect of ploidy increase is the change in gene expression, cell size, nuclear size and organ size [DEL POZO & RAMIREZ-PARRA, 2015; SLABODNICK & al. 2017; ZHAO & al. 2017]. Endoreduplication helps in gene amplification, radiation resistance and cell differentiation [BARLOW, 1978; GALBRAITH & al. 1991]. Endoreduplication is quite common in various tissues of plants like pavement cells of leaf, trichomes, sepals of flower. In fact, degree of endoreduplication is developmentally regulated in most somatic tissues in *Arabidopsis* depending on age and tissue types [GALBRAITH & al. 1991]. The cells in the

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Arabidopsis sepals exhibit a characteristic pattern, with diverse sizes ranging from giant cells of 360 μm to the smallest cells of 10 μm in length. These giant cells of flower are the markers for sepal organ identity [ROEDER & al. 2010]. Similarly, petals are of great interest to horticulturalists to enhance the ornamental value of plants [NISHIHARA & NAKATSUKA, 2011]. The petal in *Arabidopsis* seems to be simple but has a more complex and diverse cell types. It generally arises from L2 and L3 layer of SAM from where the other aerial organs arise [JENIK & IRISH, 2000]. Petal growth and size is very important for attracting the pollinators. It is composed of diverse cell types which serve as a model for morphogenesis [IRISH, 2008]. The earlier studies have shown that petal has elongated proximal cells and small distal cells. But how these cell types are different in terms of its ploidy characterization is not known. Like a leaf, petal growth also involves initial cell proliferation followed by cell expansion, thus favors the interaction between cell number and size control during petal growth [MIZUKAMI & FISCHER, 2000; SZÉCSI & al. 2006]. Is endoreduplication, a part of normal petal growth? This question has not been addressed so far. In *Arabidopsis* mutants like *frl1, p*etal cells displayed little endoreduplication at the tip [HASE & al. 2005] but endoploidy in wild type petal is not known so far. Generally, endoreduplication is mostly associated with cells that become enlarged and is an important factor for controlling the cell size [MIZUKAMI, 2001; MELARAGNO & al. 1993]. The question which has been addressed in the present study is to know the endoreduplication signals in various tissues of *Arabidopsis* and whether it is related to cell expansion or not.

This is the first study in which SAM, stem, leaf and petal cell types are taken into account for ploidy analysis by flow cytometry. In shoot apex we did not see endoreduplication however as the cells descend towards the stem or leaf, they enter into differentiation pathways and undergo endoreduplication. Also, we are able to separate the two different cell types of *Arabidopsis* petal on the basis of their cell size through florescent activated cell sorter and have shown for the first time the presence of different ploidies in them. The cells towards the distal end of petal were found to be diploid whereas elongated/elliptical cells at the proximal end of the petal have undergone endoreduplication to approx. 128C. A relationship was also proposed between ploidy levels and cell size during petal development.

Material and methods

Protoplasting and florescent activated cell sorting (FACS) of SAM cell types

For protoplast isolation, 1 cm, 4 cm and 10 cm stem from shoot tip of *WT Col* and *Ler* was cut down and chopped into small pieces into 6 ml of protoplasting cocktail. On the other hand, approximately 50 petals of 28 days old mature *Arabidopsis* (*Ler*) plants were harvested and placed in falcon tube containing 6 ml of protoplasting cocktail. This cocktail was prepared by dissolving 1.25% w/v Cellulase (Yakult), 0.3% w/v Macerozyme (Yakult), Hemicellulase (Sigma), 0.4 M D-mannitol, 20 mM MES and 20 mM KCl (from a 1 M stock) in demineralized water and adjust the pH to 5.7 with 1 M Tris/HCl pH 7.5. Further the solution was heated to 55 °C for 10 minutes (to make it clear) and cooled down to room temperature before adding 0.1% w/v BSA (bovine serum albumin), 10 mM $CaCl_2$, and 5 mM β-mercaptoethanol. The tubes were allowed to shake for about 1 hour and 15 min at 120 rpm. After shaking, the protoplast solution containing tissue was filtered through 40 μm cell strainer (BD Falcon) and the filtrate was centrifuged at 4 °C for 20 minutes at 500 g. The pellet was resuspended in 1 ml of the incubation solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM MES, adjust pH to 5.7 with KOH) and was used for flow cytometry for sorting of cells on the basis of size.

Cells were sorted on the basis of size by fluorescence activated cell sorter (BD, FACS Aria Fusion) with a 100 µm nozzle at a rate of 2,000 to 5,000 events per second and sheath fluid pressure of 20 psi. Protoplasts sorting were established based on the following cell properties: a) a cluster of live protoplasts with intact membranes was selected based on a forward to side scatter ratio. b) Doublet exclusion was performed by plotting width versus area for forward and side scatter and finally gate was applied to identify cells small and big cells and sorting was performed. About 10,000 protoplasts were collected from each sample. Cells were sorted directly in LB01 isolation buffer (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100; pH was adjusted to 7.5 before adding ß-mercaptoethanol to 15 mM) for ploidy and cell cycle analysis.

DNA Ploidy and cell cycle analysis

For DNA ploidy and cell cycle analysis, flow cytometry using DNA-selective fluorochromes method was used for the measurement of nuclear DNA content in the protoplast cells. For nuclei isolation, protoplasts sorted above in LB01 isolation buffer was incubated at dark for 15 min on ice with occasional shaking. After that homogenate was filtrated through 42 µm nylon mesh and the DNA florochrome, propidium iodide (PI) was added to the filtrate at the concentration of 50 µg/ ml simultaneously with 50 µg/ ml of RNase in order to prevent the binding of PI to dsRNA. This solution was incubated in ice for 15 min-1 hr before analysis of nuclear DNA content through flow cytometer (BD Aria Fusion).

Results and discussion

Early signs of endoreduplication observed just below shoot apical meristem in *Arabidopsis*

To explore the early sign of differentiation in search of genetic signature, we analyzed the ploidy in shoot and stem of *WT Col* and *Ler* ecotypes of *Arabidopsis* by flow cytometry. This study has revealed the specific endoreduplication pattern in stem and shoot. The shoot as a meristematic tissue found to consist of diploid 2C and 4C cells only (Figure 1a). Interestingly, the signs of endoreduplication were observed as we go down towards the stem and leaf of *Arabidopsis*. For stem, we have analyzed the ploidy in the nuclei isolated from the cells taken 1 cm, 4 cm and 10 cm from shoot tip of *WT Col* and *Ler*. The signs of endoreduplication with small peak of 8C was observed in the cells isolated from 1cm below the shoot tip (Figure 1d & 1g) in both ecotypes of *Arabidopsis*. Excited from this result, we extended the distance and analyzed the ploidy in the cells that were harvested 4 cm and 10 cm from shoot tip, respectively. 8C nuclei were found to be more abundant as distance increases down the stem (Figure 1e & 1h). Similarly, the cells with 16C were found to appear when we got down to 4cm and 10 cm in *WT Col*, *Ler* (Figure 1f & 1i). The pavement cells analyzed from leaf tissue showed endoreduplication from 4C, 8C, 16C and occasionally up to 32C and beyond (Figure 1b). The above findings suggest that indeed, ploidy is an essential mechanism that leads to stem elongation and cell growth in most plant species. The proportion of polyploid cells increases in stem as the distance increase from shoot tip. Thus, suggested that cells near the meristem maintains their genomic content closer to 2C, however, when these cells undergo differentiation in stem and leaf they start increasing their size by increasing the genomic content. The nuclear DNA content in various tissues has been depicted by histogram (Figure 1c). The results suggest that the nuclei harvested from 1 cm below the shoot tip have early signs of endoreduplication.

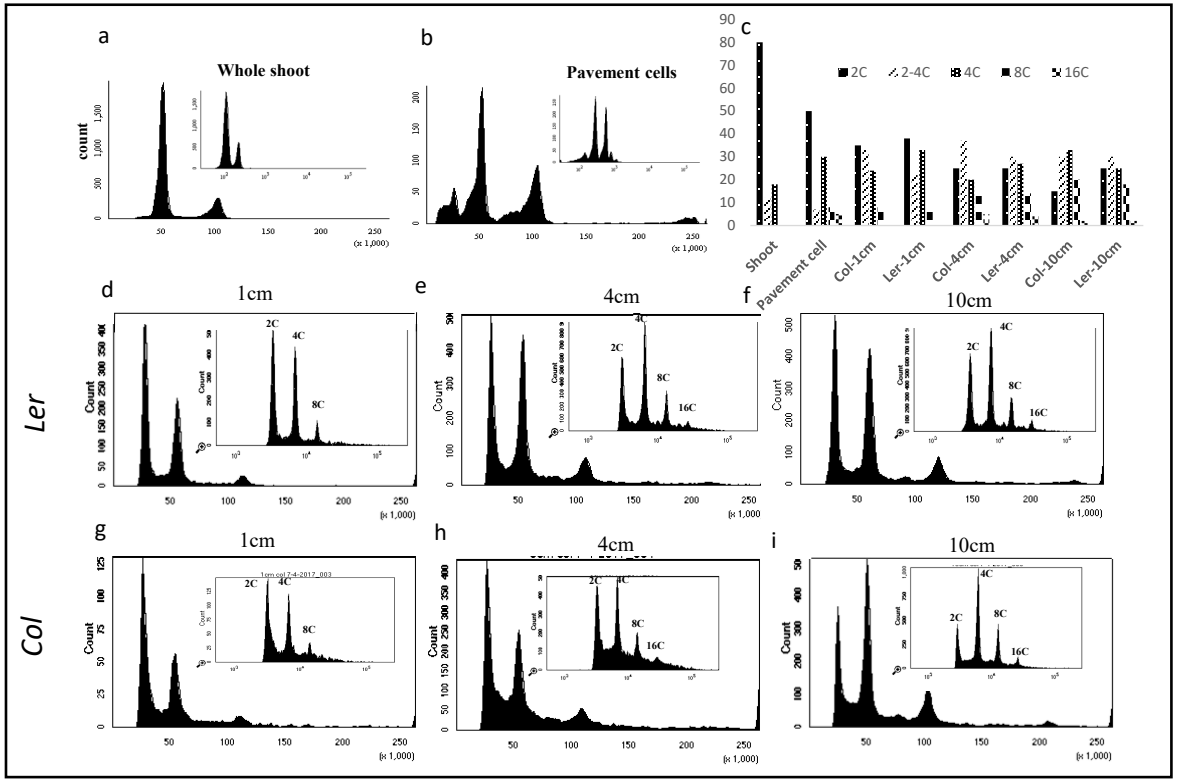

Figure 1. DNA ploidy in different tissues of *Arabidopsis*. a) Whole shoot; b) pavement cells; d) stem (1cm) down from shoot tip (*Ler* ecotype); e) stem (4 cm) down from shoot tip (*Ler* ecotype); f) stem (10 cm) down from shoot tip (*Ler* ecotype); g) stem (1cm) down from shoot tip (*Col* ecotype); h) stem (4 cm) down from shoot tip (*Col* ecotype); i) stem (10 cm) down from shoot tip (*Col* ecotype); c) Histogram showing DNA content in all the observed tissues

Endoreduplication was observed in petals

Further, ploidy was analyzed in floral petals. The protoplast was isolated from about 50 mature petals of *Arabidopsis* (Figure 2). They were subjected to FACS (BD Aria fusion) and were analyzed by taking forward scatter and side scatter parameters (area) into account. From the scatter plot, the two types of cells forming clusters were analyzed. One showing smaller cell size and the other with bigger cell size were observed. These both type of cells was sorted separately for cell cycle analysis (Figure 3). Cells sorted above on the basis of size were subjected to cell cycle analysis by flow cytometry. Nucleus was isolated and DNA content was estimated using propidium iodide. The smaller cells showed 2C and 4C peak accounting for 80% (2C) and 20% (4C) of total nuclei. Because *Arabidopsis* is a diploid species (2*n*=10), the 2C DNA level corresponds to the diploid state of the genome found in the G_1phase, whereas the 4C DNA level results from the S-phase doubling of chromatids found in the G_2phase (Figure 4a). It is thus an indicator of the capacity of cells to enter mitotic cycles. Therefore, the major 2C and 4C peaks suggest that the tissue is in a dividing state. Whereas, surprisingly, bigger cells showed a sharp peak corresponding to 128C was observed (Figure 4b). It means the cells in the proximal part of the petal during maturity have stop division and undergone endoreduplication, reaching to high ploidy level. The flow cytometric profiles displayed a reproducible 128C peak from nuclei in the proximal part of the petal at the mature stage. At this stage, in contrast, the number of nuclei with 2C, 4C, 8C, 16C, 32C and 64C DNA level decreased dramatically (Figure 4b & 4c). The study of *Arabidopsis* petals may help in providing information about the relationship between endoreduplication and cell growth during plant organ development. In the present study, one of the most striking features of petal cells observed was an uneven increase of their DNA content during development. This cytometric data showed that, in the proximal part of the petal, cells become more endopolyploid with up to a 128C ploidy level, whereas cells in the distal part of the petal maintained the diploid level throughout petal differentiation. The high proportion of nuclei at the 2C and 4C levels in distal part, indicate that the cells never endoreduplicate and their mitotic cell cycle is arrested either in the G_1 phase or in the G_2phase of the diploid cell cycle. The study has shown that the epidermal cells in the proximal part of the petal are large and extremely elongated, but epidermal cells in the distal part of the petal are small and highly homogeneous. These findings, indicated that the formation of large differentiated cells is accompanied by an increased ploidy level.

The correlation between the cell size and the degree of endopolyploidy supports the idea that the nuclear DNA content might play a key role in controlling cell size. Endoreduplication in *Arabidopsis* petals might be a major driving force for cell differentiation. According to GALBRAITH & al. (1991), endoreduplication is not present in the floral organs of *Arabidopsis thaliana*, all cells are at the 2C level [GALBRAITH & al. 1991]. But Roeder et al. 2012 have shown for the first time that sepals of *Arabidopsis* have the giant cells that have a power to undergo endoreduplication. Our data is well supported by this report of giant cell formation in the early development of sepal [LEE & al. 2009; BREUER & al. 2010]. Endoreduplication has also been documented in different organs of the cabbage flower. Filament tissue showed ploidy level up to 64C, carpel and petal showed ploidy up to 8C and 32C respectively [KUDO & KIMURA, 2001].

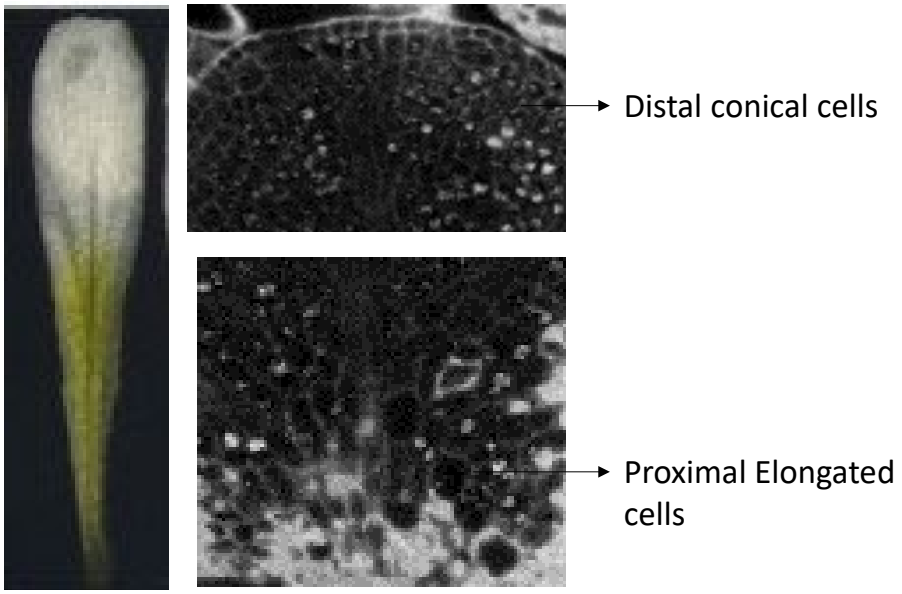

Figure 2. *Arabidopsis* flower: **A.** Morphological view of *Arabidopsis* petal taken through Nikon camera fitted to dissecting microscope. **B.** Confocal microscopic view of *Arabidopsis* petal showing the cells stained with propidium iodide.

A correlation between cell size and DNA content has also been reported in many eukaryotic organisms. Size of endoreduplicaed cells is larger than normal cells [LARKINS & al. 2001]. *Arabidopsis* mutants with increased or reduced ploidy levels in trichomes invariably showed an increase or a reduction in the final cell size [FOLKERS & al. 1997] which supports an idea that endoreduplicaton is coupled with cell size. However, some reports have shown that uncoupling also occurs in endoreduplication and cell elongation. It was observed during germination of *Arabidopsis* seedlings where endoreduplication takes place before the elongation of hypocotyl cells [GENDREAU & al. 1997]. Endopolyploid nuclei can be advantageous for specialized functions. It is proposed to increase the metabolic activity, rRNA synthesis and transcriptional activity [BALUSKA & KUBICA, 1992] like in maize and pea [CAVALLINI & al. 1995; LIU & al. 1996]. The control of endoreduplication may allow cells to reach extraordinary sizes [CEBOLLA & al. 1999]. So, the process of endoreduplication is very important for manipulating the cell size in horticultural and agronomically important crops.

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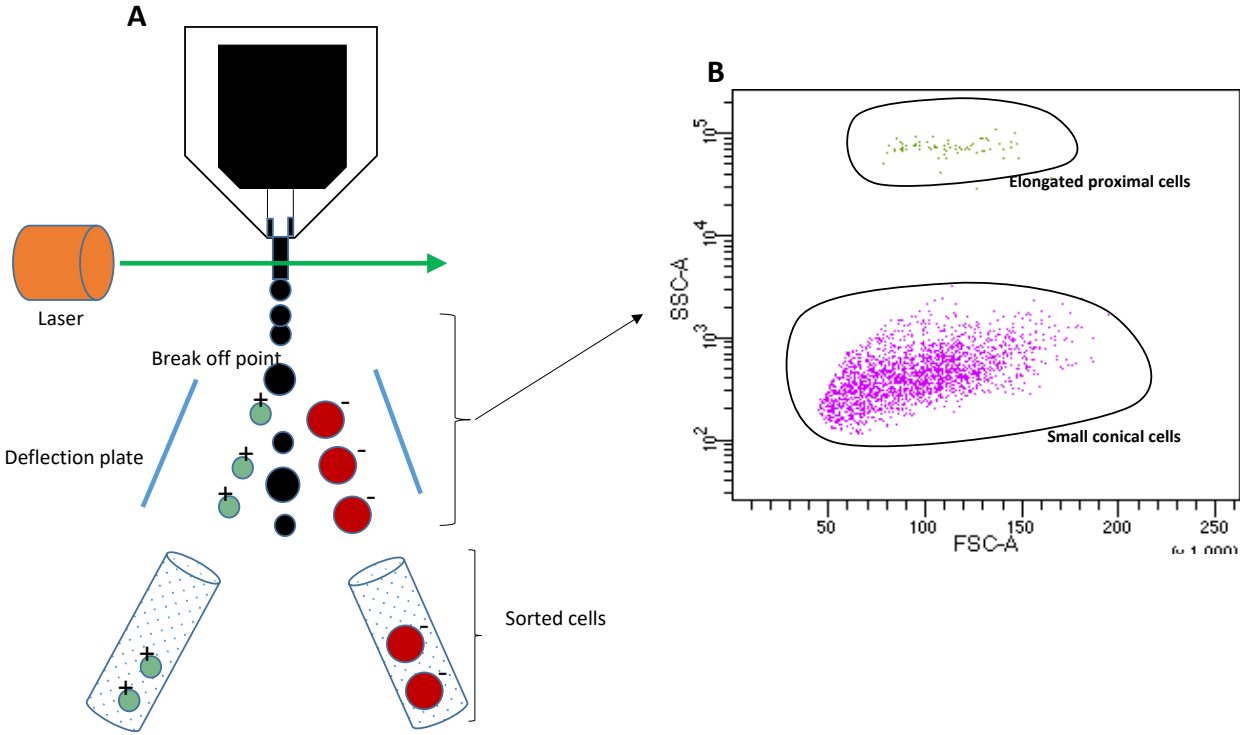

Figure 3 A. Diagrammatic representation of florescent activated cell sorter (FACS) showing the sorting of protoplast isolated from *Arabidopsis Ler* petal on the basis of their size. **B.** The scatter plot between SSC and FSC generated on the basis of size and complexity showing two types of nuclei in wild L*er* petals. Gating was done to sort these two populations separately.

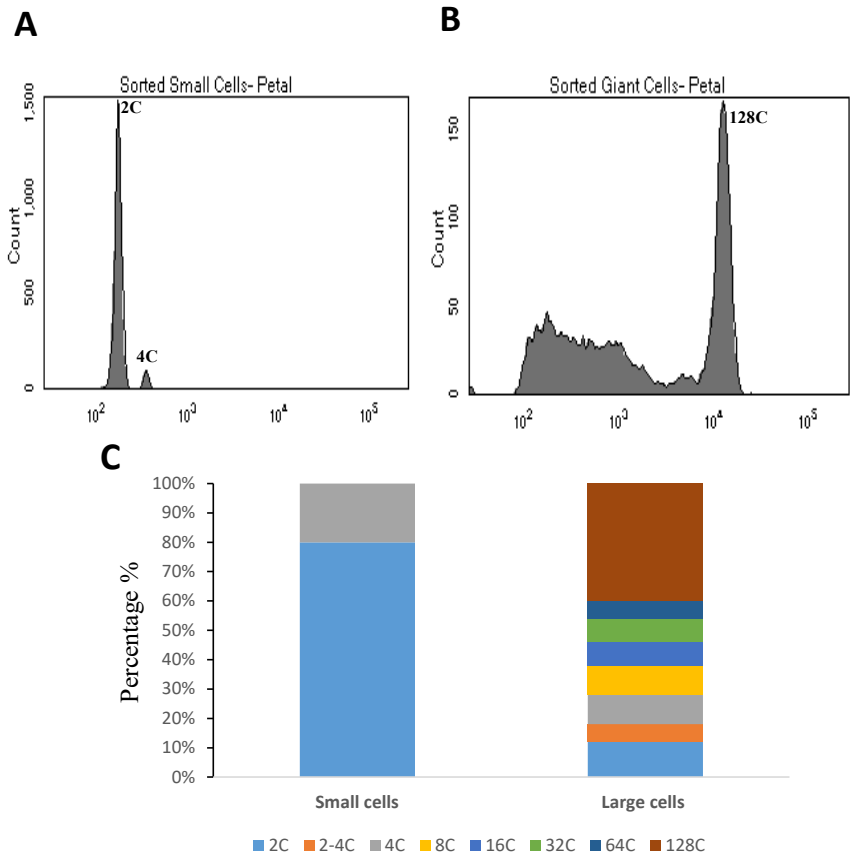

Figure 4. A. DNA ploidy in the sorted small nuclei is showing 2C and 4C peaks whereas **B.** the large nuclei is showing the higher ploidy (endoreduplication) upto 128C. **C.** Histogram showing DNA content in small and large cells of *Arabidopsis* petals.

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THE QUANTIFICATION OF SOME BIOACTIVE COMPOUNDS IN THE FRUITS OF FOUR BLACKBERRY (*Rubus fruticosus* L.) CULTIVARS, PROPAGATED BY TISSUE CULTURE

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Abstract: The goal of the research was the quantitative determination of biochemical compounds (ascorbic acid, reducing sugars, tannins and titratable acidity) in *Rubus fruticosus* L. fruits – blackberries ('Melana', 'Triple Crown', 'Arapaho' and 'Thornfree'), propagated by tissue culture and cultivated under the ecological conditions of the Republic of Moldova. The spectrophotometric determination of ascorbic acid revealed that the highest content of this phytocompound was quantified in the 'Arapaho' blackberries, 48.28 mg/100 g, followed by the 'Thornfree' cultivar (41.69 mg/100 g), which is an amount about twice as high as in the 'Melana' blackberries (23.8 mg/100 g). The high amount of vitamin C in the researched blackberries makes them comparable to raspberries and gooseberries. The content of reducing sugars varied between 4.72% and 7.26%. The 'Triple Crown' cultivar was characterized by the maximum amounts of these biochemical compounds, and the lowest amounts were found in the fruits of the 'Thornfree' blackberry cultivar. The evaluation of the titratable acidity showed that the highest index of this parameter was recorded in the 'Melana' blackberries (0.74% / 0.77% as compared with malic / citric acid. The quantification of the tannins revealed that the fruits of the 'Triple Crown' cultivar have the lowest tannin content (1.97%), the amount being more than twice lower than the maximum amount (4.16%), recorded in the blackberries of the 'Thornfree' cultivar. The results of the study led to the conclusion that the fruits of *Rubus fruticosus* L., obtained from plants which had been micropropagated in the Embryology and Biotechnology Laboratory (NBGI) and grown under the conditions of the Republic of Moldova are a good source of biologically active substances and can be proposed for commercial production as a promising organic food product.

Keywords: 'Arapaho', ascorbic acid, biochemical parameters, blackberry, 'Melana', tannins, 'Thornfree', titratable acidity, 'Triple Crown', reducing sugars.

Introduction

Rubus fruticosus L. is a perennial shrub, cultivated for its fruits, which are appreciated due to their nutritional value. The popularity of blackberries among consumers is determined not only by their pleasant appearance and delicious taste, but also by the compositional diversity of nutraceuticals they contain. Thus, the fruits of this promising crop are currently appreciated and widely used for nutritional, medicinal and cosmetic purposes.

The need to provide the market with valuable blackberries has led to the expansion of plantations, blackberry being cultivated mainly in Europe and North America. Serbia and Hungary are among the European countries with the largest areas of blackberry plantations [AFIF CHAOUCHE & al. 2015]. Wild blackberry plants also make a significant contribution to world production [STRIK, 2007].

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The health benefits of blackberries are attributed to the diversity of biochemical they contain. The results of several studies [JAMBA & CARABULEA, 2002; CANGI & ISLAM, 2003; GERCEKCIOGLU & al. 2003; KAFKAS & al. 2006; PATRAS & al. 2009; DENEV & al. 2010; ŞAHIN & al. 2010; TÜRKBEN & al. 2010; MURAD & al. 2011; DIMIĆA & al. 2012] have demonstrated that blackberries are an extremely rich source of bioactive substances, such as sugars, dietary fibre (cellulose, hemicellulose, pectin), vitamins (B group, C, E, K, P, PP), minerals (potassium salts, copper and manganese), organic acids (citric, tartaric, malic, salicylic), proteins and various macro- and microelements. In addition, blackberries are very rich in phenolic compounds, such as phenolic acids and anthocyanins, flavonoids, flavonols, ellagitannins, gallotannins and proanthocyanidins, which have shown considerable antioxidant properties [SIRIWOHARN & al. 2004; CHO & al. 2005; REYES-CARMONA & al. 2005; PANTELIDIS & al. 2007; MILIVOJEVIĆ & al. 2011; MILOŠEVIĆ & al. 2012a; GARCIA-SECO & al. 2015; KOLNIAK-OSTEK & al. 2015].

The research on the phytotherapeutic potential of blackberries has allowed attributing appreciable healing qualities to these berries. Among the diversity of healing properties of blackberries, there are the anticancer [SEERAM & al. 2006; BOWEN-FORBES & al. 2010; JIMENEZ GARCIA & al. 2013; PEREIRA & al. 2017], antitumour [KAUME & al. 2012], antimicrobial [SEERAM & al. 2012; YANG & al. 2014; AFIF CHAOUCHE & al. 2015; ČETOJEVIĆ-SIMIN & al. 2017], antioxidant [SIRIWOHARN & WROLSTAD, 2004; REYES-CARMONA & al. 2005; CHO & al. 2005; ZAFRA-ROJAS & al. 2018], antidiabetic [JIMENEZ GARCIA & al. 2013], anti-inflammatory [DAI & al. 2007; BOWEN-FORBES & al. 2010; KISS & PIWOWARSKI, 2018] and neuroprotective action [TAVARES & al. 2013], they play a role in the prevention of cardiovascular diseases [SEERAM & al. 2006; BASU & al. 2010; JIMENEZ GARCIA & al. 2013; PARMENTER & al. 2020] and are a remedy for bronchitis and respiratory infections [BLUMENTHAL & BUSSE, 1998], etc. Clinical trials have also shown the benefits of eating blackberries to reduce the risk of obesity, degenerative diseases, etc. [PEREIRA & al. 2017; KISS & PIWOWARSKI, 2018; PARMENTER & al. 2020].

The description of the broad spectrum of health benefits of blackberries has contributed to the appreciation of this crop as a highly sought after and considered healthy nutraceutical food with a significant abundance of bioactive compounds. In the Republic of Moldova, blackberry cultivation has started to be practiced in the last decade, and some varieties, such as 'Arapaho' and 'Triple Crown' have been multiplied by tissue culture in the Embryology and Biotechnology Laboratory and have been used as planting material at the establishment of modern plantations in the districts of Dubăsari, Fălești, Criuleni and Orhei. In order to evaluate the phytotherapeutic value of blackberries, obtained from plants multiplied by tissue culture and cultivated on the territory of the country and on the experimental lands of the “Alexandru Ciubotaru” National Botanical Garden (Institute) (NBGI), we decided to make a quantitative screening of important phytochemicals (ascorbic acid, reducing sugars, tannins and titratable acidity) from the fruits of different genotypes of *Rubus fruticosus*.

Material and methods

The (frozen) fruits of four genotypes of *Rubus fruticosus* L. ('Melana', 'Triple Crown', 'Arapaho' and 'Thornfree'), which had been obtained by tissue culture in the Embryology and Biotechnology Laboratory of the NBGI, served as biological material. The brief characteristic of the four blackberry cultivars [BALAN & al. 2017] and the general appearance of the fruits are presented in Table 1.

Table 1. General description of the studied blackberry genotypes

The name of the cultivar	General description	The aspect of the fruits
'**Triple Crown**'	It is a cultivar of American origin, obtained in 1998 (Maryland, USA). It has a semi-erect growth, without spines. It produces shoots about 3 m long. It blooms in May-June, being a semi-early cultivar. The ripening period lasts from early July to mid-August. The shape of the fruit is slightly elongated, the weight – 8-10 g and the yield – about 15-20 t/ha. The fruits are sweet and fragrant. They are recommended to be eaten both fresh and processed. They maintain their integrity during handling and transportation. The cultivar has average frost resistance (can withstand temperatures of -20 °C). It is a very popular cultivar and one of the best blackberry cultivars without thorns.	
'**Arapaho**'	It is a cultivar of American origin (University of Arkansas, USA). It is moderately vigorous, has no thorns, the habit is erect, with vertical growth. It blooms in May, and the ripening period lasts from early June to the end of July, being an early-ripening cultivar. The fruit has a conical shape and a weight of 6-7 g, with small seeds. Productivity is about 15 t/ha. The fruits have good organoleptic qualities. They are recommended to be eaten both fresh and processed. They maintain their integrity during handling and transportation. Mechanical harvesting is also possible. The cultivar is resistant to frost (can withstand temperatures of -24 ºC), diseases, pests and drought.	
'**Thornfree**'	It is a cultivar of American origin, very vigorous, without thorns, with long branches. It blooms in June, and the ripening period lasts from mid-August to early September. The shape of the fruit is conical-elongated, the weight is 8-9 g and the productivity of the cultivar is high (up to 20 t/ha). The fruits have a pleasant taste, are slightly flavoured. The fruits are recommended to be eaten both fresh and processed. They maintain their integrity during handling and transportation. The cultivar is resistant to frost (can withstand temperatures of -20 ºC) and to the main diseases and pests. The cultivar has been homologated in the Republic of Moldova and has been registered in the catalogue of plant cultivars.	

'Melana'	It is a cultivar obtained in the Embryology and Biotechnology Laboratory of the NBGI, which is now in the process of being approved by the Testing Commission (CSTSP), of medium size, moderately vigorous, without spines, productive. The plant has an erect and compact habit; vertical support is recommended. The fruits are conical in shape, large in size, weighing 7-8 g when ripe, blackish in colour, aromatic, maintain their integrity during handling and transportation, can be preserved well for a long time, having an attractive commercial appearance, the ripening of the berries is uniform. Ripening period – from the middle of June until the beginning of July (21-25 days). It is considered an early cultivar, which is highly resistant to frost (-24 ºC), drought, diseases and pests, suitable (adapted) to the conditions of the Republic of Moldova.	

Biochemical investigations were performed at the Institute of Genetics, Physiology and Plant Protection using different biochemical methods (spectrophotometric and titrimetric).

The quantitative determination of vitamin C. The quantification of ascorbic acid content included spectrophotometric quantification at 680 nm wavelength using potassium hexacyanoferrate. In an acidic medium, ascorbic acid reduces stoichiometrically potassium hexacyanoferrate (Fe^{+3}) $K_3[Fe(CN)_6]$ (red salt) in potassium hexacyanoferrate (Fe^{+2}) $K_4[Fe(CN)_6]$ (yellow salt), which, in the presence of ferric ions, forms *iron* (III) *hexacyanoferrate* (II) ("Berlin Blue") $Fe_4[Fe(CN)_6]_3$.

To determine the concentration of vitamin C in the plant extract, the calibration curve was used and the following formula was applied:

$$K = (49{,}967 \cdot D\ opt) - 11{,}938$$

The following formula was used to calculate the ascorbic acid content in a sample:

$$C = \frac{K \cdot V}{m}$$

where: C – ascorbic acid content, µg/g biological material; K – concentration of ascorbic acid in a ml of extract, calculated according to the calibration curve, µg/ml; V – total volume of the extract, ml; m – the weight of the biological sample, g.

The quantitative determination of reducing sugars was based on the spectrophotometric quantification of the glucose, fructose and galactose content in the aqueous plant extract. The optical density was determined at a wavelength of 582 nm. The reducing sugar content was calculated using the formula:

$$A = \frac{c \cdot V \cdot 100\%}{m \cdot 1000}$$

where: A – sugar content, %; c – glucose content determined on the basis of the calibration curve; V – the volume of the extract corresponding to the sample of plant product, ml; m – the weight of the plant product taken for analysis, g.

Determination of total titratable acidity. The method is based on titrating the test solution with a standard solution of sodium hydroxide (0.1 N) in the presence of phenolphthalein. Exact amounts of aqueous extract were titrated with sodium hydroxide solution (0.1 N) to which phenolphthalein solution (1%) had previously been added. Titration

with stirring was continued until the solution changed its colour to pink, which did not disappear for 30 seconds.

The concentration of tartaric, malic or citric acid (g/l) was determined according to the formula:

$$X = \frac{V_1 \cdot C \cdot M}{V_0}$$

where: V_0 – the volume of the sample taken for titration; V_1 – the volume of sodium hydroxide solution that was consumed during titration, ml; V_2 – the volume to which the sample was adjusted, ml; C – the exact concentration of sodium hydroxide, (0.1 g/mol); m – the weight of the sample, g; M – molar mass of citric (64.0) / malic (67.0) / tartaric (75.0) acid.

The mass fraction of titrated acids relative to tartaric, malic or citric acid (%) was determined by the formula:

$$X_1 = \frac{V_1 \cdot V_2 \cdot C \cdot M}{m \cdot V_0} \cdot 0{,}1$$

where: V_0 – the volume of the sample taken for titration; V_1 – the volume of sodium hydroxide solution (0,1 N), which was consumed during titration, ml; V_2 – the volume to which the sample was adjusted, ml; C – the exact concentration of sodium hydroxide (g/mol); m – the weight of the sample, g; M – the molar mass of citric (64.0) / malic (67.0) / tartaric (75.0) acid.

The determination of tannins in the researched biological material consisted in their quantification with potassium permanganate (0.1 N), according to the classical titrimetric method [GOST 19885-74] as a result of the process of oxidation of tannins. The calculation of the percentage of tannin content was done using the formula:

$$C\ (\%) = \frac{(a - a_1) \cdot 0{,}004157 \cdot V \cdot 100}{V_1 \cdot m}$$

where, a – the quantity of potassium permanganate consumed to oxidize the tannins in the sample; a_1 – the quantity of potassium permanganate consumed to oxidize the tannins in the control (water and indigo carmine); V – the total volume of the sample; V_1 – the volume of the sample used for quantification; m – the dry mass of the sample, g; 0.004157 – the quantity of tannins oxidized by 1 ml of potassium permanganate (0.1 N), g.

Statistical processing. The search results were analysed using the program Microsoft Excel. The average was calculated for each parameter, and the data were expressed as the average of the replicates.

Results and discussions

Blackberries have a diverse content of biologically active substances, and the quantitative assessment of the nutraceuticals present in blackberries is of particular importance in identifying the phytochemical fingerprint of these berries.

Ascorbic acid. It is an essential water-soluble vitamin with excellent reducing properties, well known for its high antioxidant activity due to the neutralization of free radicals and other reactive oxygen species, produced by cellular metabolism, which are associated with several forms of tissue damage and diseases [SKROVANKOVA & al. 2015]. Although ascorbic acid is an important antioxidant, it still provides a maximum of 10% of the total antioxidant capacity of blackberries [MÄÄTTÄ-RIIHINEN & al. 2004; CHO & al. 2005; LANDETE, 2011; LI & al. 2015]. Vitamin C also helps extending the shelf life of berries, including blackberries [ZIA-UL-HAQ & al. 2014].

The spectrophotometric quantification of ascorbic acid in the four blackberry genotypes studied by us revealed that the lowest content of this phytocompound was determined in blackberries of the 'Melana' cultivar, namely, 23.8 mg / 100 g (Figure 1). 'Thornfree' and 'Arapaho' cultivars contained amounts of ascorbic acid about twice as high as 'Melana' blackberries (41.69 and 48.28 mg / 100 g, respectively). The fruits of the 'Triple Crown' cultivars indicated values of 29.5 mg / 100 g, which is a higher content (by 24%) than the minimum value and significantly lower (by 39%), in comparison with the maximum value detected. Significant variations, which are even bigger than those recorded by us, have been obtained by other researchers. Thus, PANTELIDIS & al. (2007) mentioned that the content of ascorbic acid determined in different blackberry cultivars was between 14.3 and 103.3 mg/100 g of fresh mass. This considerable variation in the researched genotypes can be conditioned by both intrinsic and extrinsic factors.

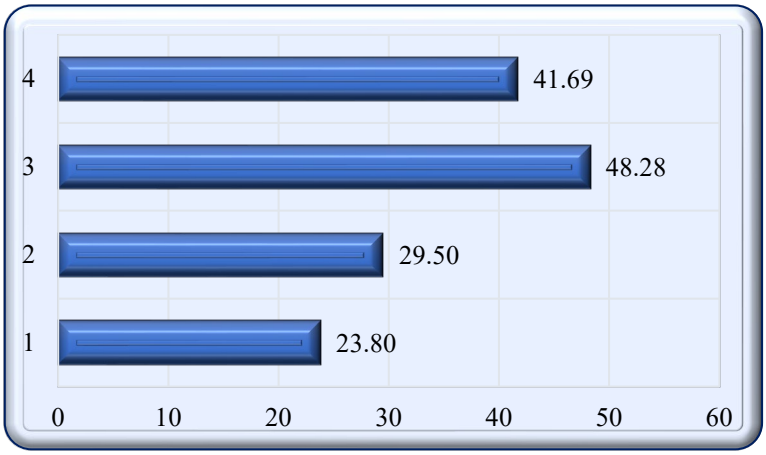

Figure 1. The content of ascorbic acid (mg/100 g) in frozen fruits of *Rubus fruticosus* L.
(1 – 'Melana'; 2 – 'Triple Crown'; 3 – 'Arapaho'; 4 – 'Thornfree')

The relatively high content of ascorbic acid in the researched fruits is similar to the results obtained by other researchers. For example, in the blackberry cultivars grown in Serbia the amounts of vitamin C varied between 35.20 mg / 100 g and 44.00 mg / 100 g [MILOŠEVIĆ & al. 2012b]. Moreover, the 'Thornfree' cultivar grown in Serbia reached values of 40.48 mg / 100 g, and the one cultivated in the Republic of Moldova and researched by us contained 41.69 mg / 100 g of vitamin C, the data being very close. The high content of ascorbic acid in the blackberries of the 'Arapaho' and 'Thornfree' cultivars is almost similar to that of raspberries (*R. ideaus*), which according to the data obtained by VELJKOVIĆ & al. (2019) is 46.62 mg / 100 g^{-1} fresh mass, and according to other authors [ARIFOVA & GORB, 2020] – from 31.7 mg / 100 g to 61.7 mg / 100 g. Also, in terms of vitamin C content, the researched blackberries are comparable to the gooseberry fruits, which according to investigations carried out on nine cultivars of *Ribes grossularia* L., grown under the conditions of the Republic of Moldova, contained between 26.51 mg% and 46.87 mg% ascorbic acid [SAVA, 2015].

The results of a recent study carried out by a group of researchers in Brazil [CROGE & al. 2019] revealed an ascorbic acid content of 20.38 mg / 100 g – 28.07 mg / 100 g, with a

variation of 9.63% depending on the place of cultivation and a variation of 8.47% depending on the cultivar, thus demonstrating a significant correlation between the vitamin C content and both the geographical region and the genotype. KULAITIENĖ & al. (2020) agree with it, they concluded that the concentration of vitamin C in berries and vegetables can be influenced by various factors, such as genotypic differences, climatic conditions before harvest, cultivation practices, maturity, harvesting methods and post-harvest handling procedures.

Reducing sugars. Sugars not only contribute to the nutritional value and the taste of fruits, but also play an important role in redox processes. All monosaccharides are reducing sugars, along with some disaccharides, some oligosaccharides and some polysaccharides [NELSON & COX, 2008], and the most common reducing monosaccharides are galactose, glucose and fructose [CAMPBELL & FARRELL, 2012]. These three monosaccharides were quantified in the fruits of the cultivars under study in order to be able to make conclusions about the reducing sugar potential of these fruits.

The content of dosed reducing sugars varied between 4.72% and 7.26%. The 'Triple Crown' cultivar was characterized by the maximum amount of these biochemical compounds, and the lowest amount was found in the fruits of the 'Thornfree' blackberry cultivar, followed by those of the 'Arapaho' cultivar, the values being close and the difference being only 1.7%. The medium content of reducing sugars was found in 'Melana' blackberries (Figure 2).

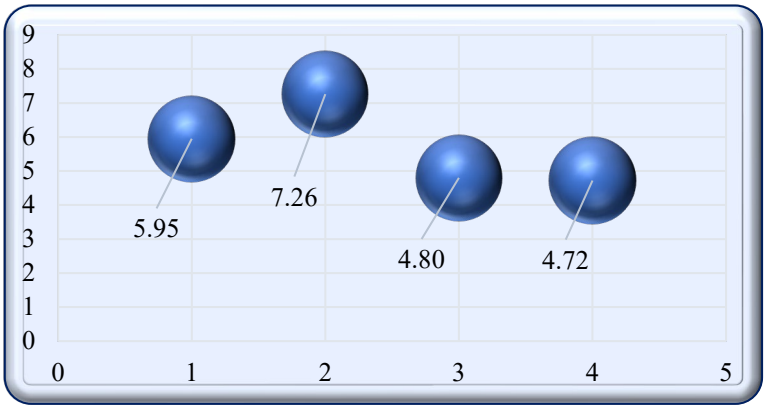

Figure 2. The content of reducing sugars (%) in frozen fruits of *Rubus fruticosus* L.
(1 – 'Melana'; 2 – 'Triple Crown'; 3 – 'Arapaho'; 4 – 'Thornfree')

The results obtained by us are comparable to the results of other studies conducted on different cultivars and even species of blackberry. A study on this parameter in the fruits of seven cultivars of blackberries grown in Serbia revealed that the content of reducing sugars varied between 5.65% and 9.08% [MILOŠEVIĆ & al. 2012b].

Another study, conducted by another group of researchers from Serbia [STAJČIĆ & al. 2012], evaluated the content of reducing sugars in the fruits of two blackberry cultivars and established much lower indices (1.32 g / 100 g and 1.46 g / 100 g fresh material). Also, lower indices (0.8 mg/g – 2.0 mg/g fresh material), as compared with our data, were obtained by a group of researchers from the USA [THOMAS & al. 2005]. These significant differences allow us to assume that geographical and pedoclimatic factors are very important for the ability of these plants to synthesize and accumulate reducing sugars.

Titratable acidity. It is well known that both sugars and organic acids are the main water-soluble substances in berries and play a crucial role in developing the taste and in the process of ripening of blackberries, as well as being a qualitative index of how they will be appreciated by consumers. Thus, organic acids together with sugars and their properties, along with various secondary and aromatic metabolites determine the taste and organoleptic properties of fruits [MIKULIC-PETKOVSEK & al. 2021]. At the same time, organic acids inhibit the development of bacteria in fruit juices and thus extend the shelf life of the product.

The results obtained during our research showed that the highest amount of titratable acidity is characteristic of the blackberries of the 'Melana' cultivar (0.74% and 0.77%, as compared with the malic acid and citric acid, respectively), and this index is approx. 30% higher than in the other three cultivars, which recorded similar results (Figure 3).

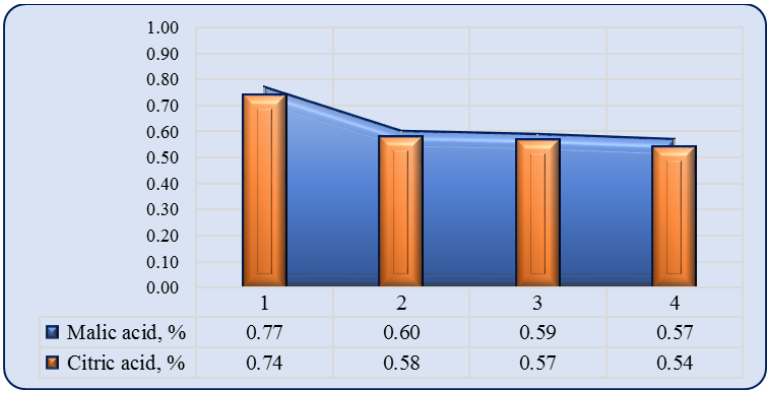

Figure 3. Titratable acidity (%, acid malic/citric) determined in the frozen fruits of *Rubus fruticosus* L. (1 – 'Melana'; 2 – 'Triple Crown'; 3 – 'Arapaho'; 4 – 'Thornfree')

In the study conducted by the researchers STAJČIĆ & al. (2012) from Serbia, which was mentioned above with reference to the content of reducing sugars of the fruits of two blackberry cultivars, much higher amounts of titratable acidity were found (1.36 and 1.39 g / 100 g material fresh).

The biochemical analysis made by YILMAZ & al. (2009) on 16 blackberry genotypes from the spontaneous flora and 9 varieties cultivated in Turkey found titratable acidity amounts between 0.5% and 1.5%, with higher amounts (about 10%) in the genotypes from the spontaneous flora. The data obtained by MILOŠEVIĆ & al. (2012a) revealed titratable acidity values in the range of 1.33%-1.89% (2010) and 1.08%-1.64% (2011), concluding that the level of this parameter differs greatly from one cultivar to another, as well as from one year to another and depends very much on the temperature during the fruit ripening period.

In another study carried out in Serbia, by a group of researchers lead by Professor Tomo Milošević, titratable acidity values between 1.69% and 2.36% were found [MILOŠEVIĆ & al. 2012b], which are much higher as compared with those obtained by us. Significantly lower values as compared with ours were obtained by a team of researchers from the USA [THOMAS & al. 2005], who evaluated the titratable acidity of the fruits collected from six blackberry cultivars, including 'Arapaho' and 'Triple Crown'. The level of this parameter varied between 0.16% and 0.34%.

The comparison of the results obtained in this study with those obtained by other researchers shows both congruence and differences between our studies and other studies in terms of titratable acidity, which may be due to climatic factors, differences in the harvesting time and total fluctuations in temperature during the growing season. The degree of maturation and ripening of the fruits also proved to be very important. In a study conducted in the USA [SIRIWOHARN & al. 2004] it was established that blackberries (*Rubus* L. hybrids) harvested in different stages of ripening can have a very variable level of titratable acidity, from 0.47 g / 100 g up to 2.38 g / 100 g, and the minimum value was obviously characteristic of overripe fruits. Thus, the multitude of factors that influence the titratable acidity are the reason why significant differences in the level of this biochemical parameter were recorded in various studies.

Tannins. Tannins are polyphenols, which have therapeutic properties and act as antioxidants, thus, they have various pharmacological properties, such as antitoxic, anticancer, antiallergic and anti-inflammatory, anthelmintic, antimicrobial, antiviral etc. [GHOSH, 2015]. They have the ability to combine with proteins, forming impermeable and non-rotting compounds. Simultaneously with the precipitation of proteins, a retraction of the tissue takes place, reducing the surface of action, a property used particularly in the treatment of burn wounds. Due to the action of protein precipitation, tannins also possess antiseptic properties, preventing infection by inhibiting bacterial growth. Naturally, tannins are present in leaves, seeds, bark, roots, fruits and vegetables [HASSANPOUR & al. 2011; GHOSH, 2015].

In our research, it was found that the 'Triple Crown' cultivar had the lowest tannin content (1.97%), the amount being more than twice lower than the maximum amount (4.16%), recorded in the blackberries of the 'Thornfree' cultivar. As in the case of reducing sugars (Figure 2), 'Melana' blackberries showed a moderate tannin content (2.81%) as compared with the other three cultivars (Figure 4).

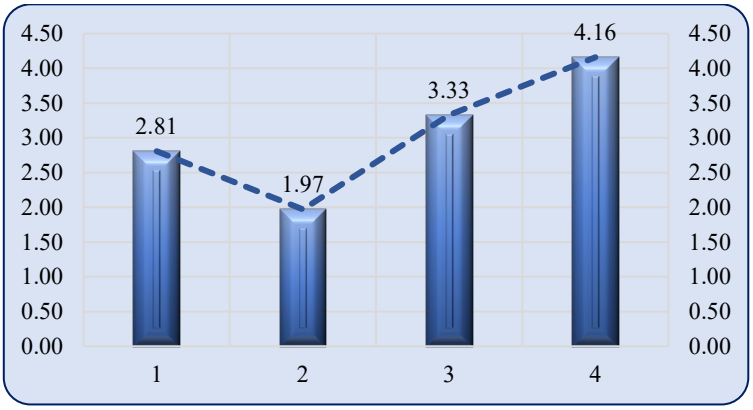

Figure 4. The percentage of tannins in frozen fruits of *Rubus fruticosus* L.
(1 – 'Melana'; 2 – 'Triple Crown'; 3 – 'Arapaho'; 4 – 'Thornfree')

A previous study conducted in the laboratory indicated a tannin content of 1.19%-6.16%, quantified in blackberries – *Rubus fruticosus* and *Rubus candicans* [LOZINSCHII, 2019]. The maximum value was identified in the blackberry fruits of *R. candicans* from the spontaneous flora.

There is very little data on the content of tannins in blackberries in literature, and the existing ones differ greatly from one study to another and depend primarily on the cultivar and the harvesting time. More research has been done on the content of tannins in blackberry leaves.

The generalization of the obtained results by their comparative analysis and the classification of the cultivars depending on the content of the quantified substances, allowed us to conclude that the four cultivars separated into two groups with relatively similar indices (Figure 5).

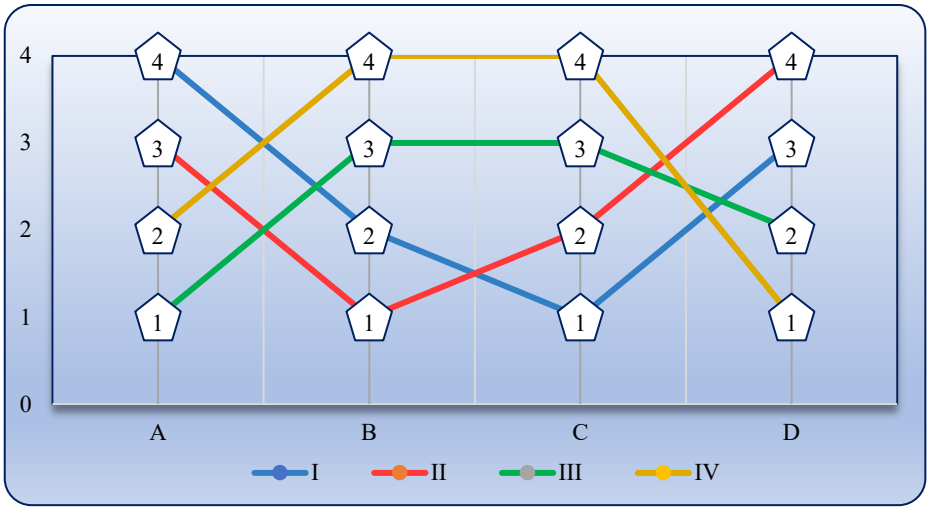

Figure 5. The classification of blackberry genotypes, according to the values of the researched biochemical parameters (1-4 – ranking by content in descending order (1 –1st place, with maximum value, 4 – 4th place, with minimal value); A-D – biochemical parameters (A – ascorbic acid, B – reducing sugars, C – titratable acidity, D – tannins); (I-IV – blackberry cultivars (I – 'Melana', II – 'Triple Crown', III – 'Arapaho', IV – 'Thornfree')

The first identified group includes the genotypes 'Melana' and 'Triple Crown' and is characterized by a higher content of ascorbic acid and tannins and lower amounts of reducing sugars and titratable acidity. The second group includes the 'Arapaho' and 'Thornfree' cultivars with a higher level of reducing sugars and titratable acidity and with a lower content of vitamin C and tannins.

Conclusions

The spectrophotometric quantification of ascorbic acid has revealed very different values depending on the genotype, ranging from 23.8 mg / 100 g ('Melana') to 48.28 mg / 100 g ('Arapaho'). The recorded values indicate a considerable amount of vitamin C in blackberries that makes them comparable to raspberries and gooseberries. The quantitative analysis of the reducing sugars revealed a maximum content of 7.26% in the fruits of the 'Triple Crown' cultivar and a minimum one (4.72% and 4.8%) – in the 'Thornfree' and 'Arapaho' cultivars. The mass concentration of titratable acids expressed in malic/citric acid revealed values

between 0.54% and 0.77%. The highest level was determined in the 'Melana' blackberry fruits, and the other three cultivars had similar values, which turned out to be about 30% lower than the maximum. The evaluation of the tannin content revealed maximum values (4.16%) in the 'Thornfree' cultivar, and the minimum tannin content (1.97%) was quantified in the 'Triple Crown' blackberries.

The comparative analysis of the obtained data has resulted a phytochemical outline, in which the four genotypes were separated into two groups with relatively similar indices. The first identified cluster includes the genotypes 'Melana' and 'Triple Crown' and is characterized by a higher content of ascorbic acid and tannins. The second group includes the 'Arapaho' and 'Thornfree' cultivars, with a higher level of reducing sugars and titratable acidity. The results of the study led to the conclusion that the fruits of *Rubus fruticosus* L. ('Melana', 'Triple Crown', 'Arapaho' and 'Thornfree'), obtained from plants which had been micropropagated in the Embryology and Biotechnology Laboratory and grown under the conditions of the Republic of Moldova are a good source of biologically active substances and can be proposed for commercial production as a promising organic food product, with impressive health benefits. At the same time, we would like to mention that the climate of the Republic of Moldova is suitable for the cultivation of *Rubus fruticosus* L., which is a crop that prefers a mild climate.

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NUTRITIONAL AND EXTRACTABLE OIL PROFILE IN SEEDS OF *SESAMUM INDICUM* L. AND *MORINGA OLEIFERA* LAM. GROWN IN SOKOTO, NIGERIA

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Abstract: Nutritional and extractable oil profile in seeds of *Sesamum indicum* L. and *Moringa oleifera* Lam. were investigated using standard biochemical procedures. Proximate analysis revealed % crude protein contents of 24.32% in *S. indicum* while *M. oleifera* had 27.66%. Crude lipid contents were analyzed and 47.78% was obtained in *S. indicum* while 28.87% was obtained in *M. oleifera*. Crude carbohydrate analysis revealed *S. indicum* with 37.89% while in *M. oleifera*, it was 34.51%. Crude fibre obtained was 11.32% in *S. indicum* while 9.37% was identified in *M. oleifera*. Higher ash content of 9.13% was obtained in *M. oleifera* while 7.62% was obtained in *S. indicum*. Available energy (k/cal.) was analyzed in the samples with obtained values in *S. indicum* 692.22 k/cal. While *M. oleifera* had 545.91 k/cal. With significant difference (P≤0.05) between the two species in terms of available energy (k/cal.). Results of extractable oil profile of *S. indicum* and *M. oleifera* revealed appreciable amounts of the oil with 54.65% found in *S. indicum* while *M. oleifera* had 39.33% with significant difference (P≤0.05) between the two species. Physico-chemical properties of the seed oils analyzed include, acid value determined with 34.32 mg $KOHg^{-1}$ for *S. indicum* and 29.98 mg $KOHg^{-1}$ obtained in *M. oleifera*. Saponification value of the two samples indicated that *S. indicum* had 148.82 mg KOH/g while *M. oleifera* had 127.88 mg KOH/g. Kinematic viscosity was determined and *S. indicum* had 0.97 mm^2/s while 0.78 mm^2/s was identified in *M. oleifera*. Iodine value was determined and *S. indicum* had 128.56 g l_2 /100 g while *M. oleifera* had 103.68 g l_2/100 g. Specific gravity was determined with 0.89 g/cm3 obtained in *S. indicum* while 0.84 g/cm^3 was obtained in *M. oleifera*. Cetane number was determined; *S. indicum* had 34.00 while 30.00 was obtained in *M. oleifera*. Oil colour was determined and the colour ranged from yellowish-brown to creamy-yellow for *S. indicum* and *M. oleifera* respectively with no significant difference (P≤0.05) in iodine number, acid value, kinematic viscosity and cetane number. State of the oil at room temperature indicated that the oils from the two seed types are liquid at room temperature. Mineral analysis of the two samples indicated that they comprise of appreciable amounts of minerals with phosphorus 385.51±4.96 mg / 100 g obtained in *M. oleifera* while in *S. indicum*, 254.54±4.06 mg / 100 g was obtained. Calcium was richly obtained in the two samples with 95.20 mg / 100 g obtained in *M. oleifera* while 66.70 mg / 100 g was obtained in *S. indicum*. However, potassium, manganese, copper, and magnesium were appreciably contained in the seeds with significant difference (P≤0.05) between the two samples. Thus, it can be recommended that seeds of *M. oleifera* especially and that of *S. indicum* should be properly incorporated in the diets especially in the developing countries where hunger and malnutrition ravage the growing children and pregnant women.

Keywords: Biofuel, minerals, *Moringa*, proximate-composition, *Sesamum.*

Introduction

Sesamum indicum L., commonly known as beniseed, sesame, is herbaceous plant grown in tropical countries such as Nigeria, India, China, Sudan, Burma, Bangladesh, Indonesia, Egypt and Tunisia. The species belongs to the family Pedaliaceae. Sesame seed has one of the highest

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oil contents of any seed and is considered to be the oldest oil seed crop known to man, highly resistant to drought and is annual crop [DUTTA, 2004]. Its seed which contains approximately 5% of oil and very high quality (47% oleic acid and 39% linoleic acid) and 25% protein aseptically high in methionine and tryptophan. The oil is widely employed in cooking and in manufacture of margarine; antioxidant, beta-carotene, and steroids have also been found in Beniseed oil. The seeds have been valued throughout history for their contributions to diet (in snacks and as soup ingredient) medicine, industry and household use [YOSHIDA & al. 2001]. Beniseed is a cherished soup condiment in some parts of Nigeria-northern states, middle belt and parts of cross river state in Nigeria. The plant's root and leaves are used for treating migraine, hypertension, ulcers, constipation, chicken pox and piles [ODUGBEMI, 2006]. Beniseed could be regarded as one of the most ancient oil seed cultivated known to mankind [OKUDU & al. 2016]. It is a highly priced oil crop of some countries of the world; its seeds are used extensively in Asia and African because of its high contents of edible oil and proteins [MAKINDE & AKINOSO, 2019].

S. indicum grows in a well-drained soil; it survives standing water or high salinity environments. The plant is notable for its ability to grow under drought conditions and in extreme heat. It is often grown where cotton can grow, under conditions few other crops can survive, requiring very few water inputs. These attributes make the species an excellent candidate for low input sustainable food systems. *S. indicum* is deep-rooted and will scavenge nutrients from below most crop root zone. Generally, the plant will have a better chance of survival when it is grown in hotter than optimal temperatures rather than lower than optimal temperatures. Sesame ranked 2nd and 7th in the world in terms of beniseeds production, and Nigeria is one of the major producers of sesame and it is also among the key commercial crops in Nigeria [NAERIS, 2010].

Moringa oleifera Lam. belongs to the family Moringaceae which is single genus in the family of shrubs and trees cultivated across the whole of the tropical belt and used for a variety of purposes. *M. oleifera* is a native to Africa, Arabia, South Asia, South America, Sub Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan, and it is the most widely cultivated out of the fourteen species in the family. *M. oleifera* is a small, fast-growing evergreen or deciduous tree that usually grows up to 10 to 12 m in height, with open crown of dropping fragile branches, feathery foliage of trip innate leaves and thick corky, whitish bark. When grown in soils, *M. oleifera* grows rapidly reaching high height; however, it can tolerate sandy soils, clay soils and water-limited conditions. *M. oleifera* is not a nitrogen fixing tree but its fruits, flowers and leaves all contain 5% to 10% protein on average. All of those parts are eaten widely as vegetable; providing excellent food for both human and animals. *M. oleifera* could be described as a monogenetic plant in the family of Moringaceae and it has long being cultivated and all its parts are being consumed and used for a variety of purposes [JAHN, 1984]. This is because of its impressive range of nutritional and medicinal values [BUKAR & al. 2010]. More so, OLUDURO (2012) reported the presence of the following minerals in the leaves sodium 11.86, potassium 25.83, calcium 98.67, magnesium 107.56, zinc 148.56, iron 103.75, manganese 13.55 among others in parts per million and nutrients such as carbohydrate 45.43%, proteins 16.15%, fats 9.68%, crude fibre 9.68%, moisture 11.76% and ash 10.64%.

The dry seed suspension is known to be a natural coagulant and coagulant aid. In northern Nigeria, the fresh leaves are used as a vegetable, roots for medicinal purposes and branches for demarcation of property boundaries and fencing. The seeds is instead have attracted scientific interest as *M. oleifera* seed kernel contains a significant amount of oil (up to 40%) with a high-quality fatty acid composition on (oleic acid >70%) and after refining, a notable

oxidative degradation. Moreover, after oil extraction, the seed cake can be used in waste water treatment as a natural coagulant or as an organic fertilizer to improve agricultural productivity. According to the Food and Agricultural Organization's (FAO) report, 70-80% of world's population especially in developing countries, relies on herbal medicine to prevent and cure diseases. In recent years, bio-energy source have become more important as available and economically alternative to diminishing and much expensive fossil fuels.

The rapidly growing global demand for petroleum products and the consequent depletion of the crude reserves in addition to adverse environmental concerns and unstable nature of the international market make imperative the need to explore alternative sources of fuel. Biodiesel stands to be the key promising renewable energy options already exploited by various countries. Categories of feedstock as source of suitable oil for biodiesel production include seeds, nuts, leaves, wood, and even bark of trees. Nigeria is very well endowed with various edible and non-edible oils [IBETO & al. 2012].

Materials and methods

Sample collection and preparation

Ripe pods of *Moringa oleifera* were sourced from the Garden of Government Girls' College within Sokoto metropolis while newly harvested seeds of Beniseed were sourced from the orchard in Sokoto, Sokoto state. The seeds were taken to the Departmental Herbarium, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto, where voucher specimens were deposited. The seeds were thereafter sun dried and seeds of *Moringa oleifera* were dehulled by removal of the shell in order to obtain the seed kernels. Sesame seeds were sorted out to remove good seeds from bad ones. Both the kernels of *M. oleifera* and Sesame were crushed using pestle and mortal and put in sterilized labeled bottles until used.

Extraction procedure for *indicum* and *Moringa oleifera* using Soxhlet extractor

Extraction of the Cucurbits seed oil. Extraction procedure

Adopting the method as reported by [AJIBOLA & al. 2018], two hundred (200) g of air dried and pulverized seeds of each of the sampled seeds will be weighed and packed into thimble, which will in turn be placed into Soxhlet extractor. The extraction solvent (n-hexane 500 cm^3) and anti-bumping chips are to be put into 1000 cm^3) round bottomed flask and heated on heating mantle at 60 °C. The extraction will be allowed to continue for one hour (1 hr). The solvent in the round bottomed flask will be collected and concentrated in vacuo using a rotatory evaporator at 40 °C. The above process will be repeated to get means of percentage extraction and enough oil for further analyses.

Percentage yields was calculated for each of the two samples using the equation bellow:

$$\text{Biodiesel yield (\%)} = \frac{\text{weight of the biodiesel}}{\text{weight of the sampled oil}} \text{x } 100$$

Physicochemical properties of the seed oil

Determination of the saponification value

The American Standard for Testing and Material (ASTM) method-[D 5558-95] was employed for the determination of the saponification values of the vegetable oil. The oil (5 g) was weighed into Erlenmeyer flask and 0.5 M ethanolic KOH to be prepared by dissolving 7 g of KOH in 250 cm ethanol and 25 cm^3 of the prepared 0.5 M ethanolic KOH was added and the resulting mixture refluxed for 60 minutes. The resulting solution was subsequently titrated

against 0.5 M HCl by diluting 10.7 cm HCl in 250 cm^3 of distilled water using phenolphthalein as indicator. The resulting end points was obtained when the pink colour changed to colorless. The same procedure was used for the blank. The Saponification value (SV) was calculated using the following expression:

$$\text{Saponification value (S.V.)} = \frac{5.61\ (B-S)\ x\ M\ of\ \ HCl}{\text{Weight of Sample}}$$

where, B – vol. of HCl required by blank, S – vol. of HCl required by sample. M – molarity of HCl, 5.61– molar mass of KOH.

Determination of acid value

Acid value of the oil will be determined by ASTM method (ASTM – D 974). The oil (0.5 g) of the oil will be weighed into 250 cm3 conical flask and 50 ml of neutralized ethyl alcohol was added, prepared by neutralizing a solvent mixture of 25 cm^3 5.61 (B-S) x M of HCl Weight of sample ethanol and 25 cm^3 diethyl ether with 0.1M ethanolic KOH was prepared by dissolving 1.4 g KOH in 250 cm^3 of ethanol using phenolphthalein as indicator. The mixture was added to the oil and heated on a water bath to dissolve the oil. The solution was then titrated against 0.1 M KOH prepared by dissolving 1.4 g of KOH in 250 cm of distill water using phenolphthalein as indicator. The acid value was determined after which the free fatty acid was respectively calculated using the following equations:

$$\text{Acid Value} = \frac{A\ x\ Mx\ 56.10}{W}$$

where, A = ml of 0.1M KOH consumed by sample, M = Molarity of KOH, W = weight in grams of the sample.

Determination of iodine value

The oil (0.5 g) will be weighed into conical flask and 20 $cm^3$of carbon tetrachloride was added to dissolve the oil. 25 $cm^3$of Wigs reagent was added into the flask using a measuring cylinder in a fume chamber and a stopper was inserted, the content of the flask was vigorously swirled and kept in the dark for 35 minutes. 20 cm of 10% aqueous potassium iodide was prepared by diluting 10 cm^3 of potassium iodide in 90 cm^3 of distilled water was added into the content of the flask using a measuring cylinder. The content was titrated with 0.1 M sodium thiosulphate solution prepared by dissolving 3.95 g of anhydrous $Na_2S_2O_3$ in 250 cm^3 of distilled water. Few drops of 1% starch indicator were added and the titration continued by adding the sodium thiosulphate drop wise until coloration disappeared after vigorously shaking. The same procedure was used for the blank test. The Iodine Value (I.V.) is given by the expression:

$$\text{Iodine Value (I.V.)} = \frac{126.9\ C\ (V1 - V2)}{M}$$

where, C = concentration of sodium thiosulphate, V_1 = volume of sodium thiosulphate used for blank, V_2 = volume of sodium thiosulphate used, M = mass of sample while 12.69= constant.

Determination of specific gravity

Specific gravity bottles will be washed, rinsed with acetone and dried at room temperature in a desiccator and the weight of the empty bottles determined using an electronic weighing balance. The weight of the bottle filled with water will also be recorded. The same procedure will be repeated with the oil and the specific gravity computed as follows;

$$\text{Specific gravity} = \frac{W2 - W1}{W3 - W1}$$

where, W_1 = weight of empty bottle, W_2 = eight of bottle + oil, W_3= weight of bottle + water.

Colour and physical state of oil at room temperature

The oil colour was determined by Oganonetip method, where ten people were called up to visualize the physical appearance of the biodiesel [AOAC, 1975]. While physical state of the oil was determined by sensory evaluation [IBETO & al. 2012].

Determination of the cetane number of the biodiesel

This is a measurement of the combustion quality of diesel fuel during compression ignition. The cetane number of the biodiesel was calculated via the use of empirical formula in the literature using the result of saponification number (SN) and the iodine value (IV) of biodiesel [AOAC, 2000].

Proximate composition analysis

Proximate composition (crude proteins, crude lipids, fibre, moisture and ash) of the seeds of the sampled cucurbits were determined using the methods of [AOAC, 1990] while carbohydrate was determined by difference. The calorific values in kilo joule (k) were calculated by multiplying the crude fat, protein and carbohydrate by Atwater factors of (k) 37, 17, and 17 respectively.

Mineral composition analysis

The minerals were analyzed by first dry ashing the samples at 550 ºC in the muffle furnace. The filtered solutions were used to determine Na, K, Ca, Mg, P and N by means of atomic absorption spectrophotometer (AAS). Phosphorus was determined calorimetrically by using the phosphovanado molybdate method [AOAC, 2008].

Data Analysis

Treatments were replicated three times and results have been presented as means ± S.D. of the values. The results obtained were subjected to one way Analysis of Variance (ANOVA). Same superscripts means that there was no significant difference ($P \leq 0.05$) and where the superscripts differ, it means that there was a significant difference ($P \leq 0.05$).

Results and discussion

Percentage yield and physiochemical properties of the seed oil of *S. indicum* and *M. oleifera*

The table below gives the results of biodiesel yield (%) and its physico-chemical properties for sampled *S. indicum* and *M. oleifera* seeds. Percentage moisture of the two seed oils revealed 3.66% obtained in *S. indicum* while 4.23% was obtained in *M. oleifera*. The yield of seed oil was both appreciable with 54.65% obtained in *S. indicum* while in *M. oleifera*, 39.33% was obtained with significant difference between the two species. Acid value of 34.32 $mgKOHg^{-1}$ was obtained in *S. indicum* while 29.98 $mgKOHg^{-1}$ was obtained in *M. oleifera*. Moisture Percent 3.66±0.54 was obtained in *S. indicum* while 4.23±0.87 was obtained in *M. oleifera*. For *S. indicum,* 148.82 mg KOH/g was obtained as the saponification value while 127.86 mg KOH/g was that obtained for *M. oleifera*. Iodine value obtained in *S. indicum* was

128.56 g I_2/100g while in *M. oleifera*; it was 103.68 g I_2/100g. For kinematic viscosity 0.97 mm^2/s was obtained in *S. indicum* while 0.78 mm^2/s was obtained in *M. oleifera.* Cetane number, 34.00was obtained in *S. indicum* while 28.00 was obtained in *M. oleifera*. Oil colour in the two samples was found to be yellowish-brown and cream-brown while state of the oil at room temperature was liquid. There was significant difference ($P \leq 0.05$) between the two species in terms of % yield, saponification values and iodine value. Obtained results are in close range to the reported yields of 58.60 and 48.40 as reported by [KARAYE & al. 2020] on Beniseeds and watermelon respectively. Acid value measures the presence of corrosive free-fatty acids and oxidation products. Percentage oil yield obtained in the current study is a bit higher than the reported 36.7% on seed oil of calabash however; obtained specific gravity is in agreement with the reported values by [SOKOTO & al. 2013]. In another study by [IBETO & al. 2012], the following results reported on the seed oil from *Brachystegia eurycoma*, *Cucurbita pepo* and *Luffa cylindrica* were comparable to obtained results in the current study.

Table 1. Physiochemical properties of the seed oil of *S. indicum* and *M. oleifera*

Parameters	**Unit**	***S. indicum***	***M. oleifera***
Moisture	(%)	3.66 ± 0.54^a	4.23 ± 0.87^a
Oil yield	(%)	54.65 ± 2.56^a	39.33 ± 1.10^b
Acid Value	$mgKOHg^{-1}$	34.32 ± 1.67^a	29.98 ± 1.16^a
Saponification value	(mg KOH/g)	148.82 ± 1.78^a	127.86 ± 1.43^b
Kinematic viscosity	(mm^2/s)	0.97 ± 0.07^a	0.78 ± 0.09^a
Specific gravity	g/cm^3	0.89 ± 0.05^a	0.84 ± 0.05^a
Iodine value	(g I_2 /100 g oil)	128.56 ± 2.23^a	103.68 ± 2.19^b
Cetane number	-	34.00 ± 0.47^a	28.00 ± 0.45^a
Oil Colour	-	Yellowish brown	Cream yellow
State of Oil at Room Temp.		Liquid	Liquid

Results have been presented as means ± S.D. of the means. The results obtained were subjected to one way analysis of variance (ANOVA). Same superscripts means that there was no significant difference ($P \leq 0.05$) and where the superscripts differ, it means that there was a significant difference ($P \leq 0.05$).

Proximate Composition of the Seeds *S. indicum* and *M. oleifera.*

Proximate compositions of the seeds of *S. indicum* and *M. oleifera* have been shown in Table 1. From the results, there was significant difference ($P \leq 0.05$) between the two species in the contents of crude lipids, crude fibre, % ash and available energy (kcal/100 g). From the Table, crude protein obtained in *S. indicum* 27.66±1.23% was a bit higher than that of *M. oleifera* with 24.32±1.08% while crude lipid obtained indicated that *S. indicum* had 47.78±1.89% while in *M. oleifera*, it was 28.87±1.12%. For crude carbohydrate, 37.89±1.52% was obtained for *S. indicum* while 34.51±1.35% was obtained in *M. oleifera*. Crude fibre revealed that *S. indicum* had 8.32±0.88% and *M. oleifera* had 14.37±0.98%. Percentage ash contents and available energy showed that *S. indicum* had 5.62±0.76% and 692.22±4.45 k/cal while *M. oleifera* had 15.13±0.97% and 545.91±3.15 k/cal respectively. Results obtained in the current study is a bit higher than the report of [KARAYE & al. 2021] on three Nigerian cucurbits seeds with the range of values of crude proteins, crude lipids and crude carbohydrates as 32.66-35.94%, 24.50-31.33% and 24.06-36.34% respectively. Results obtained in the current study is in disagreement with the report of [NZIKOU& al. 2009] the seeds of *S. indicum* grown in

Congo-Brazzaville with moisture contents, proteins, carbohydrates and crude fibre as 5.7%, 20%, 13.4% and 3.2% respectively.

Table 2. Proximate composition of the seeds *Sesamum indicum* and *Moringa oleifera*

Parameters	*Sesamum indicum*	*Moringa oleifera*
Moisture	4.14±0.18[a]	3.32±0.14[a]
Crude Protein (%)	27.66±1.18[a]	24.32±1.12[a]
Crude Lipid (%)	47.78±1.89[a]	28.87±1.12[b]
Crude Carbohydrate (%)	37.89 ±1.52[a]	34.51±1.36[a]
Crude Fibre (%)	8.32±0.88[a]	14.37±0.98[b]
Ash (%)	5.62±0.76[a]	15.13±0.97[b]
Available Energy (K Cal.)	692.22±4.45[a]	545.91±3.15[b]

Results have been presented as means ± S.D. of the means. The results obtained were subjected to one way Analysis of Variance (ANOVA). Same superscripts means that there was no significant difference (P≤0.05) and where the superscripts differ, it means that there was a significant difference (P≤0.05).

Mineral analysis of the seeds of *S. indicum* and *M. oleifera*

Mineral compositions of the samples were shown in Table 2. Minerals are important in human nutrition. It is a well-known fact that enzymatic activities as well as electrolytic balance of the body fluid are related to adequacy Na, K, Mg and Zn. Potassium is very important in maintaining body fluid volume and osmotic equilibrium, the pH of the body, regulation of muscles and nerve irritability, control of glucose absorption and enhancement of normal retention of protein during growth [ADESINA & ADELEYE, 2016]. From the Table 3, it can be deduced that the two species contain appreciable amounts of valuable nutrients needed for healthy growth and development. Phosphorus contents in the samples revealed that 385.51 mg / 100 g was obtained in *M. oleifera* while it was 254.54 mg / 100 g obtained in *S. indicum*. Potassium is the next in abundance with 198.32 mg / 100 g obtained in *M. oleifera* while in *S. indicum*; 157.97 mg / 100 g was recorded. Calcium is another vital mineral obtained in both the seeds with appreciable composition of 198.32 mg / 100 g obtained in *M. oleifera* while 157.97 mg / 100 g was recorded in *S. indicum*. Magnesium is the next in the series with 144.96 mg / 100 g] obtained in *M. oleifera* while in *S. indicum*; 118.13 mg / 100 g was recorded. Sodium is the next in abundance with 135.87 mg /100 g in *M. oleifera* while 112.43 mg / 100 g was obtained in *S. indicum*. Manganese is the other vital elements contained by the two seeds with 112.14 mg / 100 g obtained in *S. indicum* while the higher value 147.83 mg / 100 g was obtained in *M. oleifera*. Cupper is the other vital elements contained appreciably by the two seeds with 124.13 mg / 100 g obtained in *S. indicum* while 145.87 mg /100 g was obtained in *M. oleifera*. Zinc is other vital element obtained in the seeds with (12.87 mg / 100 g) in *S. indicum* while in *M. oleifera*; 31.84 mg / 100 g was obtained. The present result is lower than that reported in another study by [OKUDU & al. 2016] with sodium 235.74 mg / 100 g, calcium 428.78 mg / 100 g and magnesium 184.12 mg / 100 g. Obtained results were however; lower than the reported 466.03 mg / 100 g, 184.12 mg / 100 g, 428.78 mg / 100 g and 235.74 mg / 100 g as for potassium, magnesium, calcium and sodium by BORCHANI & al. (2010). However, it has been reported that climatic factors and stages of maturity could cause variation in distribution of the phytochemicals [BAMISHAIYE & al. 2011].

Table 3. Result of mineral analysis of the seeds of *Sesamum indicum* and *Moringa oleifera* using (AAS) presented as mg / 100 g

Minerals	*Sesamum indicum*	*Moringa oleifera*
K	157.97±2.12[a]	198.32±3.80[b]
Mg	118.13±1.48[a]	144.96±3.66[b]
P	254.54±2.06[a]	385.51±4.96[b]
Ca	166.70±1.85[a]	295.20±2.61[b]
Mn	112.14±1.31[a]	147.83±1.19[b]
Na	121.43±2.13[a]	145.87±2.56[b]
Cu	124.13±1.08[a]	162.81±2.63[b]
Zn	12.87±1.09[a]	31.84±1.67[b]
Ni	3.95±0.51[a]	16.07±1.68[b]
Cr	3.32±0.11[a]	2.22±0.21[a]
Cd	0.03±0.09[a]	0.28±008[a]

Results have been presented as means ± S.D. of the means. The results obtained were subjected to one way Analysis of Variance (ANOVA). Same superscripts means that there was no significant difference (P≤0.05) and where the superscripts differ, it means that there was a significant difference (P≤0.05).

Conclusion

To conclude, it can be asserted that the two seed samples are endowed with multiple benefits that if incorporated into daily diets of the populace, they could go a long way in providing succor to the fight against hunger and malnutrition especially in the developing world. This is in addition to the fuel they contain that could play a vital role in providing income to the poor populace.

Conflict of interest

Authors hereby declare that there is no competing interest of any sort among them.

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INFLUENCE OF SOIL TYPE ON GROWTH AND ARTEMISININ CONTENT OF WORMWOOD (*ARTEMISIA ANNUA* L.) CHEN YOUNG VARIETY IN SOKOTO

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Abstract: *Artemisia annua* L. produce an array of complex secondary plant metabolite including artemisinin (ART), which kills the principal malarial parasite, *Plasmodium falciparum*, a compound of current interest in the treatments of drug resistant malaria. However, this compound remain expensive and hardy available on global scale. Synthesis of ART has been proved to be economically impossible. Therefore, increase in yield of natural occurring ART is necessary. The study evaluated the influence of soil types on germination, growth and artemisinin content of *A. annua* of Chen Young variety in Sokoto agro ecological region of Nigeria and. A greenhouse experiment was conducted in 2017 at Botanical garden of Usmanu Danfodiyo University Sokoto. The seeds were sown in plastic pots containing clay, sandy and loamy soils in completely randomized design with 4 replications. Germination percentage (G), Mean germination time (MT), Coefficient of variation of the germination time (CV_t), Mean germination rate (MR), Uncertainty of germination (U) and Synchrony of germination (Z) were evaluated. Growth parameter and Artemisinin content were also determined. ART was determined and quantified with high–performance liquid chromatography (HPLC) using calibration curve constructed by plotting the peak area against the concentration (5, 10, 15, 20, 25 µg/ml) of ART standard solutions. The results revealed that soil types had no significant effect on germination and growth parameters evaluated ($P<0.05$). However, clay had the highest G, MT and CV_t than sandy and loamy soils. The highest speed of germination per day was observed on sandy soil. Clay soil had the least values of U (2.277±0.2 bit) while the degree of overlapping germination was frequent in the clay (Z). The result obtained shows that treatment had no significant effect on the growth parameters evaluated ($P<0.05$). Artemisinin content was significantly affected by soil type with the higher content in sandy soil (37.73 µg/ml) follow by clay and loamy soils with 17.90 and 15.70 µg/ml respectively. This study concludes that *A. annua* seeds can germinate and survived on different soils type in Savanna region of Nigeria. The study suggested the use of sandy soil in cultivation of the plant for its influence on high artemisinin content.

Keywords: *Artemisia annua*, artemisinin, germination, high performance liquid chromatography, soil type.

Introduction

Artemisia annua belongs to the family Asteraceae (Compositae) and is the second largest family of the flowering plant in the world. *Artemisia* is a large, diverse genus with between 200 and 400 species and comprises of hardy herbs and shrubs. *A. annua* is an annual shrub of 50-150 cm in height. It grows in temperate climate, and is most widespread in China and Vietnam, but it is also cultivated in east Africa, United States, Russia, India, Brazil and some other countries [BHAKUNI & al. 1988; 1990; LESTARI & al. 2011]. It is characterized with extreme bitterness and has been used in China for over 2000 years to treat fever [The State Pharmacopoeia of people Republic of China, 1985]. *A. annua* contains a natural chemical called

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artemisinin, which was isolated in 1972 [HIEN & WHITE, 1993] and kills the principal malaria parasite, *Plasmodium falciparum*.

Artemisinin is a complex secondary plant metabolite which cannot be economically synthesized *de novo*. The unique configuration of the oxygen atoms in this molecule make it difficult to synthesize and this is key to its potent anti-malarial activity. Artemisinin is thought to work through generation of a carbon-centered free radical, which interferes with more than one biochemical pathway important in the growth and infection process of *P. falciparum* parasites. This complex mode of action, which could be said to 'starve' the parasite, may also help to limit the buildup of resistance. The World Health Organization (WHO) has recommended artemisinin-based therapies in combination with another effective blood schizontocide (such as mefloquine) to reduce recrudescence and to slow the development of resistance. More than fifty countries have now adopted *A. annua* – based anti-malaria as the front line treatment for multi-drug resistant malaria.

Artemisinin is a sesquiterpene lactone that is produced and stored in the glandular trichomes that are mainly on the leaves and floral buds of *A. annua* [DUKE & al. 1994; DUKE, 2001; FERREIRA & al. 2005]. The plant also produces more than forty flavonoids [FERREIRA & al. 2010], many polyphenols and a variety of other terpenes including mono-, sesqui-, di-, and triterpenes [BHAKUNI & al. 2001]. Many of these have weak anti-malarial activity, and, based on transcriptome analyses, many also seem to be produced and/or stored in the glandular trichomes that also contain artemisinin [WANG & al. 2009].

In low income and developing nations, malaria is the fifth most prevalent infectious disease and the tenth overall cause of death, and is projected to remain at that level until at least 2030 [MATHERS & LONCAR, 2006]. The WHO estimated that more than 229 million cases of malaria occurred in 2019 and accounting for more than 386,000 deaths in Africa [WHO, 2020]. The development of resistance by *P. falciparum* to many classes of anti-malarial drugs made the World Health Organization (WHO) to recommend artemisinin-based combination therapy (ACT) as first-line treatment of Malaria [WHO, 2018].

A. annua is a medicinal herb (Asteraceae) used for the production of artemisinin, a sesquiterpene lactone with anti-malarial effects against susceptible and multi-drug resistant *Plasmodium* species [TIRUNEH & al. 2010]. Although, artemisinin can be synthesized chemically, the analogues are unlikely to be economically competitive with that produced in plant due to the high cost and complexity of the process [FERREIRA & al. 2005]. In spite of the huge economic burden borne by many countries in the tropics, especially those within sub-Saharan Africa as a result of malaria, little is known about the agronomic performance of *A. annua* in most of these areas. Empirical reports indicating its introduction in a few countries in Africa such as Cameroon, Ethiopia, Kenya, Mozambique, Tanzania, Uganda and Zambia are available all in high-altitude regions and/or regions with a pronounced cool period [MAGALHÃES & al. 1997, MUELLER & al. 2004; EABL, 2005; FERREIRA & al. 2005]. In Nigeria, successful propagation of *A. annua* has been reported by EBIAMADON & al. (2012) in Cross River, south-eastern Nigeria. Consequently, the drug is in short supply leading to the scarcity of ACTs (presently the most effective treatment for malaria) and needed in countries where the disease is endemic [BRISIBE & al. 2008].

For these reasons, the availability, accessibility and affordability of *A. annua* remain a problem especially in sub-Sahara Africa where malaria is endemic. Therefore, the improvement in naturally occurring artemisinin through good agricultural practice of *A. annua* is the only possible alternative at a moment. Despite this limitation to fulfil the growing demand of the drug worldwide, there is no information on the germination, growth and artemisinin content of *A. annua* grown in Savanna region of Nigeria.

Combinations of artemisinin and other anti-malarial drugs, such as mefloquine or lumefantrine, have been proven to be highly effective against the multidrug-resistant *Plasmodium falciparum* [PRICE & al. 1996; VAN VUGT & al. 2000]. In addition to this, artemisinin and its derivatives have been shown to be effective against a number of viruses, *Pneumocystis carinii*, *Toxoplasma gondii*, a number of human cancer cell lines [EFFERTH, 2007], tuberculosis [ZHENG & al. 2017], diabetes [LI & al. 2017] and a variety of other parasitic tropical diseases including schistosomiasis [UTZINGER & al. 2001], leishmaniasis [SEN & al. 2007], Chagas disease and African sleeping sickness [MISHINA & al. 2007]. Recently Madagaska's Institute of Applied research has produced Artemisia-containing tonic that supposedly prevent and treat COVID 19 [RAJOELINA, 2020]. All these diseases probably can be successfully treated with artemisinin and Artemisia plant if enough of the drug is made available and also affordable for developing countries. There is still a worldwide shortage of the drug just for treating malaria let alone other diseases against which artemisinin is promising [DE RIDDER & al. 2008]. It is estimated that the worldwide area needed to meet the current Boston Consulting Group (BCG) estimated demand for 275 million ACTs for 2015 is 23,000 hectares, based on the estimate that 1 ha^{-1} of *Artemisia* produces enough artemisinin for approximately 25,000 adult courses of ACT [NAS, 2004]. Producing artemisinin from *A. annua* is currently the only economical alternative, thus its availability is limited by low plantation. To meet the artemisinin demand in Africa, African Botanicals have to expand area and region of planting the plant. As a result, the expansion in regions under cultivation of the plant for the production of naturally occurring artemisinin through the good agricultural practice is desirable. Information on the adaptability of *A. annua* to Sokoto agro ecological zone of Nigeria is not available and hence the important for the current study.

The aim of this study is to evaluate the influence of soil types on growth and artemisinin content of *Artemisia annua* in Sokoto, Nigeria.

Material and methods

Study area

The study was conducted in the Department of Biological Science of Usmanu Danfodiyo University Sokoto. The main campus lies between latitude 13º06'-13º08' N, longitude 5º11'-5º12' E and altitude of 351.0 m above the sea level. It has about 70-125 days of rainy season and long dry season throughout the year. The mean annual rainfall is 700 mm per annum (SERC - Sokoto Energy Research Centre, 2016).

Collection of plant materials and soil

Seeds of *Artemisia annua* Chen Young variety were sourced from Artemisia Programme Unit at the Institute for Agricultural Research (I.A.R.) Ahmadu Bello University, Zaria. Three different soil types used in this research were; clay, sandy and loamy soils. Clay was obtained from University research farm in Kwalkwalawa while sandy and loamy were obtained from Biological Sciences garden of Usmanu Danfodiyo University and Nakasari area of Sokoto state respectively.

Experimental design and germination studies

The experiment was designed to investigate the influence of three different soils on germination, growth and Artemisinin contain of *A. annua*. Treatment consisted of three soils types replicated four times in a completely randomized design. The soil was potted and

replicated four times and in each pot one hundred and fifty seeds were sown (150). Random sampling was applied to both the experimental media and test plants. Transparent wire cage was used to cover the germinating media (sands) against pest and the cage was covered with transparent polythene bag to maintain adequate moisture, temperature and humidity levels in the soil which are essential for Artemisia seeds germination. The physical and chemical properties of the soil used in the experiment are presented in Table 4.

Germination studies

The seeds obtained from pilot test were collected and sown on March 20, 2017 and observation was made at 24 hours interval (a day) after sowing. Data collected include the time in days (t_i) of sowing, number of seeds germinated at each observation (n_i). The expressions for the most important germination parameters according to RANAL & al. (2009) were considered, these include; germination capacity (germinability or seedling emergence), time spent to germinate or emerge (mean germination time), speed (mean germination rate), homogeneity (coefficient of variation of the germination time), uncertainty and synchrony.

Germinability was calculated according to the formulae cited by LABOURIAU & VALADARES (1976):

Percentage germination:

$$G = \left(\frac{N}{A}\right) \cdot 100$$

where, G = percentage of germination; N = number of germinated seeds; A = total number of seeds sowed.

Mean germination time was calculated by the expression:

$$\bar{t} = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

where, t_i: time from the start of the experiment to the i^{th} observation (day for the example); n_i: number of seeds germinated in the i^{th} time (not the accumulated number, but the number correspondent to the i^{th} observation), and k: last time of germination.

Variance of germination time was calculated by the expression:

$$s_t^2 = \frac{\sum_{i=1}^{k} n_i (t_i - \bar{t})^2}{\sum_{i=1}^{k} n_i - 1}$$

where, t: mean germination time; t_i: time between the start of the experiment and the i^{th} observation (day for the example); n_i: number of seeds germinated in the i^{th} time, and k: last time of germination. The variance value will be used to calculate the CV_t.

Coefficient of variation of the germination time is calculated by the expression:

$$CV_t = \frac{S_t}{\bar{t}} 100,$$

where, s_t: standard deviation of the germination time and t: mean germination time. Replications or samples with only one seed germinated do not have the value of this measurement because the divisor of the variance of the germination time is zero.

Mean germination rate is the average speed of germination was calculated as the reciprocal of the mean germination time:

$$V = \frac{1}{t}$$

where, V = average speed of germination; t = mean germination time.

Uncertainty of germination was calculated by the expression:

$$U = -\sum_{i=1}^{k} f_i \log_2 f_i, \text{ being } f_i = \frac{n_i}{\sum_{i=1}^{k} n_i}$$

where, n_i: number of seeds germinated on the i^{th} time, and k: last day of observation.

Synchrony of germination was calculated by the expression:

$$Z = \frac{\sum_{i=1}^{k} C_{n_i,2}}{C_{\sum n_i,2}}, \text{ being } C_{n_i,2} = n_i(n_i-1)/2$$

being $C_{ni} = n_i(n_i-1)/2$, where $C_{ni,2}$: combination of the seeds germinated in the i^{th} time, two by two, and n_i: number of seeds germinated in the i^{th} time.

Z is the quotient between the sum of the partial combinations of the number of seeds germinated in each t_i, two by two and the two by two combination of the total number of seeds germinated at the end of the experiment, assuming that all seeds that germinated did so simultaneously. While waiting for the seeds to germinate, the soils were kept moist and damp by regular watering.

Effects of soil type on growth and artemisinin content in *A. annua*

To evaluate the effects of soil type on growth and artemisinin content, three different soils of the same quantity (15 kg) were potted namely loamy, sandy and clay. The three treatments were replicated four times and laid out in Completely Randomized Design (CRD). Four weeks old seedling of 12 cm height were transplanted to the experimental pots and observed for seven months. The following morphological characters were measured: plant height, length of the longest branch, branch numbers and days to flowering.

Plant height and length of the longest branch were measured in centimeter using thread and meter ruler. Branch number were numerically counted, days to flowering was taken by counting the days taken for the plant to flowers.

After harvesting, root length and fresh weight are measured. Fresh weight was measured using weighing balance (L.P 202) and the biomass was sun dried until constant weight was obtained, as experience shown that natural sun drying produces high artemisinin content [ZHANG & YU, 1997]. The dry weight of the sample was also measured. The sun dried plant sample was divided into leaves, stem and roots which were grinded using mortar and pestle, and the ground powder was used for Soxhlet extraction.

Extracts preparation

Fresh leaves of *A. annua* L. were dried for two weeks, pulverized into powder using mortar and pestle and homogenized. Adopting the method of CHRISTEN & VEUTHEY (2001), 5 g of pulverized sample was extracted with 200 mL of *n*-hexane at 60 °C in Soxhlet apparatus. The hexane was then evaporated under a vacuum and the samples reconstituted in 10 ml acetonitrile then filtered through whatman filter paper.

Preparation of standards and high performance liquid chromatography

Standard artemisinin was purchased from Sigma-Aldrich (Germany). Artemisinin solution was obtained by dissolving 10 mlg in 100 ml of acetonitrile to form the stock solution, from the stock solution, five different concentration of 5, 10, 15, 20 and 25 µg/ml were formed. Each was run 3 times in HPLC, from the result, Standard calibration curve was obtained by plotting the peak area against the concentration of ART standard solutions.

The HPLC analyses were performed with Agilent Technologies 1200 series on Eclipse XBD-C18 (4.6 x 150 mm), column and detection was conducted at 214 nm wavelength. The acetonitrile was used as a mobile phase with 0.8 ml/min flow rate [LAPKIN & al. 2009]. Injection column was 10 µL; run time of 20 min, temperature 30 °C. The gradient HPLC-UV method is widely recommended and used for quantification of artemisinin purity and amount in plant material.

Calibration curve

The calibration curve was constructed by plotting the peak area against the concentration. The Concentrations ranging between 5 and 25 µg/ml of artemisinin were prepared from the standard stock solution by serial dilution with acetonitrile for the calibration data. The calibration curves were obtained by the external standard method on five levels of concentration of standard (5, 10, 15, 20, and 25 µg/ml), with three injections per level. Linear regression was used to establish the calibration curve. Results were calculated using the peak areas with determination coefficient (R^2) was 0.951.

Soil analyses

Some of the physico-chemical properties of soil samples analysed include particle size distribution, organic C, total N, available P, exchangeable bases, soil pH and CEC. Particle size analysis was carried out using Boyoucos hydrometer method as described by GEE & BAUDER (1986). The textural class was determined using USDA soil textural triangle. Soil pH was determined with a glass electrode pH meter 1:2 soil:water ratio [BATES, 1954]. Total N was determined using micro-Kjedahl method as described by NELSON & SOMMERS (1982). Available P by Bray No. 1 Method [BRAY & KURTZ, 1945] and exchangeable bases were determined for calcium and magnesium using EDTA titration method as described by DEVIS & FREITAS (1970) and potassium using flame photometry [RICH, 1965].

Data analysis

The data obtained were analyzed using one-way analysis of variance with Minitab Statistical Software vision 17. Significant means were compared using Turkey simultaneous test at $P<0.05$.

Results and discussion

Germination of *A. annua* seeds on all the soil types tested was successful as they germinated promptly when subjected to conditions which are normally regarded as suitable for germination indicating that they are not dormant. The seeds do not require to be artificially subjected to some physical and chemical pre-sowing treatment in order to overcome the dormancy and prematured period as uniform, rapid and synchronous germination was observed in this study with 73.6, 56.6 and 56.33% germination on clay, loamy and sandy soils respectively (Table 1). This finding was in disagreement with previous report that chemical dormancy due to phenolic secretion on the seed of *A. annua* was overcome by the use of Gibberellic acid [BEWLEY & MICHAEL, 1994; NICOLAS, 2003; FAROUK & al. 2008].

Twenty four (24) hours after sowing there was no record of germination. However, germination commenced 48 hours after sowing on all the soil types at the same time. This confirms the result of MÜLLER & BRANDES (1997) that seeds of *A. annua* germinated at the same time on different soil types tested. JAMALEDDINE & al. (2011) reported that 80-90% germination of *A. annua* started 5^{th} to 7^{th} days after seeds inoculation and addition of hormones (BA and NAA) do not alter the duration of germination (Info net biodivision, 2010). At Zaria, Kaduna State, research by TAHIR & al. (2013) showed that germination of *A. annua* seeds commenced 3 days after inoculation and 50 to 60% seeds germinated to plantlets.

The early germination observed may be attributed to the high temperature of the state (average 29 °C and relative humidity 14% in January, 2017), which is essential for *Artemisia* seeds germination. Similarly, HARTMANN & al. (1997) reported that temperature is the single most important factor in the regulation of the timing of germination, because of its role in dormancy control and/or release, or climate adaptation.

The highest germination count was observed on day 4 with 33.5, 37.5 and 16.6% seedlings on loamy, clay and sandy soils respectively. Germination was recorded last on day 9 on loamy and day 10 on clay and sandy soils respectively. The first pair of leaves appeared or opened between 4 to 5 days after germination. This agreed with the WHO report that appearance of the first pair of leaves commenced between 4 to 5 days in Kenya and Tanzania. Two weeks after germination, the second set of leaves began to appear. Five days after the appearance of the second set of leaves, the third set appeared.

Germination measurements of *Artemisia annua* seeds

The results of germinability (G), Mean germination time (MT), Coefficient of variation of the germination time (CV_t), Mean germination rate (MR), Uncertainty (U) and synchrony (Z) of the germination of *A. annua* are presented in Table 1. The results showed that soil type has no significant ($P>0.05$) effect on all the parameter evaluated (G, MT, CV, MR, U and Z). However, clay soil was the most effective treatment in enhancing germinability with 73% seeds germinated, followed by 56 and 55% on loamy and sandy soils respectively. This may be attributed to the ability of clay soil to support the plants by its favorable water retention and its available macro and micro nutrients compared to loamy and sandy soils and neutral soil pH as earlier reported by ABOU HUSSIEN & al. (2010) (Table 5). Artemisia seeds require light and uniformly high level of moisture at temperature of 18-20 °C. This discovery contrasts the finding of SCHÜTZ & al. (2002) that observed germination to commence earlier and faster in sandy soil than loamy. However, MÜLLER & BRANDES (1997) stated that germination and growth of *A. annua* was limited in sandy soil as it reaches the average height of 24 to 66.5 cm. Seeds sown in clay soil generally had the highest emergence percentage and mean germination time

as compared with the seeds sown in sandy and loamy soils (Table 1) but the difference in germination percentage was not significant (P>0.05). The coefficient of variation of the germination time was proposed to evaluate the germination uniformity or variability in relation to the mean germination time and was applied to seeds of *Myracrodruon urundeuva* Allemão [DORNELES & al. 2005] and *Anacardium humile* A. St.-Hil. [CARVALHO & al. 2005]. Sandy soil had the highest coefficient of variation of germination time, a direct interpretation on the sense that high values would be associated with concentrated germination in time (seedlings weremore uniform in size). Thus, the sandy soil had more frequent germination over time than clay and loamy but means comparison showed that they were not significantly different (P>0.05). The rate of germination is also quite higher on sandy soil than clay and loamy soil with means values of 0.2264, 0.2102 and 0.1839 (Table 1). The results revealed that the rate at which seeds germinated was significantly the same (P<0.05) when compared with other soil types.

Uncertainty of germination(*U*)measures the degree of spreading of germination through time and can be used, by inference, to measure the synchrony of germination. Low values of *U* indicate frequencies with few peaks, that is, germination more concentrated in time. Only one seed germinating changes the value of *U*. Germination was more uncertain in clay soil than loamy and sandy soil because it had the least mean values of 2.277 bit and high value of mean germinated time. Frequency with high peaks in germination occurs most in sandy soil hence it had the highest value of *U* (2.424 bit). Statistically however, *U* values appear to be the same when compared between the treatments.

Synchrony of germination (*Z*) produced a number if and only if there are two seeds finishing the germination process at the same time. Thus, itmeasuresthe degree of germination overlapping). *Z* is zero when no overlapping was observed for *n* germinated seeds and will be null when no seed could complete the germination process [RANAL & al. 2009]. Clay soil had the highest overlapping emergence among the treatment followed by loamy and sandy soil. Mean comparison showed that degree of germination overlapping was thesame among the treatment (P<0.05) using Turkey simultaneous test.

Table1. Germination measurements (mean ± standard deviation) of *Artemisia annua* seed on different soil types

Soil type	G (%)	MT(day)	CV_t (%)	MR (day^{-1})	U (bit)	Z
Clay	73.8±21.3 [a]	4.803±0.6 [a]	35.44±1.8 [a]	0.2102±0.02 [a]	2.2777±0.24[a]	0.2474±0.06 [a]
Sandy	56.0±10.3 [a]	4.454±0.5 [a]	34.68±2.9 [a]	0.2264±0.02 [a]	2.4246±0.20[a]	0.2080±0.04 [a]
Loamy	55.5±21.8 [a]	4.413±0.1 [a]	24.37±12.4 [a]	0.1839±0.08 [a]	2.2909±0.14[a]	0.2340±0.04 [a]

G – germinability, MT – Mean germination time, CV_t – Coefficient of variation of the germination time, MR – Mean germination rate, U – Uncertainty and synchrony of the germination (Z). Means followed by the same superscript in each column are not significantly (P>0.05) different based on the Tukey test.

Effect of soil type on growth of *Artemisia annua*

The effect of soil type on plant height of *A. annua* is presented in Figure 1. The result revealed that soil types do not shows any significantly (P>0.05) affect on plant height. However, height of *A. annua* grown in sandy soil was higher than those grown in clay and loamy soils respectively during the first eight weeks after planting (WAP). At the tenth week, loamy soil had the highest plant height with 50.40 cm followed by sandy and clay with 49.20 and 48.60 cm soil respectively. This result corroborates with the finding of OMER & al. (2013) who reported that

A. annua grown under clay loamy soil had the highest vegetative growth parameters than those in sandy loamy soil. Clay soil had the highest plant height at 14 and 16 WAP while the lowest mean value was observed in sandy (88.80) at 16 WAP. In general, sandy soil had the best of plant height with the highest mean values of 22.60, 30.20, 35.80, 40.00, 60.80 and 71.40 at 2, 4, 6, 8 and 12 weeks respectively. Plant height in all the soil types significantly increased with the plant age to reach its maximum at flowering after 150 days and recorded 114, 108 and 97 cm in clay, loamy and sandy respectively. These values obtained were lower than those recorded by OMER & al. (2013) at 210 days (371 and 247 cm in clay loamy and sandy loamy soil). Similarly, MUELLER & al. (2000) stated that *A. annua* grow rapidly into a larger plant at higher altitudes (2000 m) where they reach the average height of 2.5 m in 7-8 month after germination.

Mean number of branches of *A. annua* grown in three different soils is presented in Table 2. The result shows that soil types had no significant (P>0.05) effect on the number of branches. This finding contradict the report of OMER & al. (2013) who noted that loamy soil produces the highest number of branches (58) than those grown in sandy loamy soil with 51 branches. The numbers of branches increased significantly between first and second month and remain the same in third and fourth month on clay and loamy soil.

The result of the longest branch length of *A. annua* plant is presented in Table 2. The result revealed that no significant (P>0.05) differences were observed between the experimental groups and length of the longest branch of *A. annua* in October and November. However, loamy soil had the highest branch length than clay and sandy in third and fourth month respectively, means comparison shows that treatment has significantly (P>0.05) effect on the longest branch length of *A. annua* plant with the highest mean on loamy (43.0), clay (34.0) and sandy (28.7) respectively. The longest branch length of *A. annua* increased significantly with the progress of plant age.

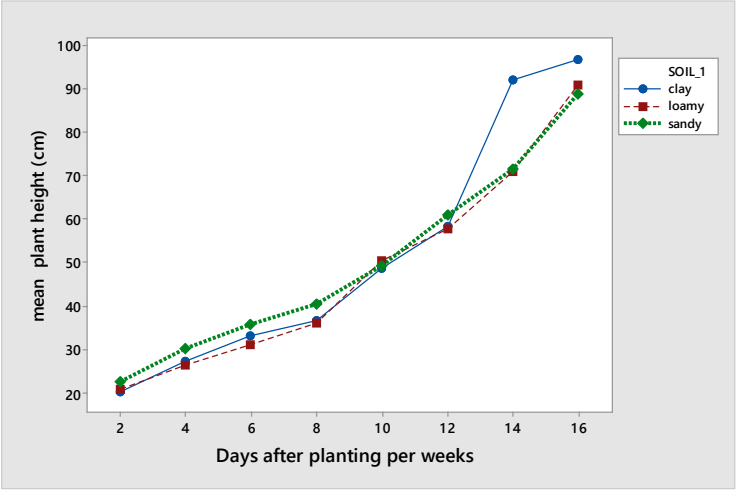

Figure 1. Effect of soil types on plant height of *A. annua*

Table 2. Effect of soil types on the number of branches and longest branch in *A. annua*.

		Sampling period (Month)			
Parameters	**Treatment**	**1**	**2**	**3**	**4**
No. of Branch	Clay	18.00 ± 4.12	23.20 ± 4.21	24.80 ± 4.02	26.20 ± 3.27
	Loamy	22.20 ± 7.82	27.00 ± 7.62	27.40 ± 6.07	29.40 ± 6.23
	Sandy	23.80 ± 11.90	27.80 ± 9.81	28.40 ± 9.10	31.00 ± 9.59
	SE	5.41	5.08	4.26	4.40
Branch length	Clay	16.20 ± 6.10	23.60 ± 3.21	29.00 ± 4.53^{ab}	34.80 ± 6.69^{ab}
	Loamy	17.60 ± 7.44	25.50 ± 7.72	34.80 ± 6.30 ^{a}	43.00 ± 11.75^{a}
	Sandy	13.40 ± 4.22	25.60 ± 1.52	26.80 ± 2.17^{b}	28.75 ± 0.957^{b}
	SE	3.83	3.06	2.92	5.48

Replication × 5. Means followed by same superscript in a column are not significantly different (P>0.05). SE; Standard error.

Effects of soil types on days to flowering

The number of days to flowering is presented in Table 3, the result shows that soil types had no significant effect on days to flowering in *A. annua* plant (P>0.05). However, sandy soil had the longest period to flower followed by clay and loamy with 150, 147 and 145 days respectively, in respect to days to flowering, sandy soil will produces the highest biomass than clay and loamy soils since the longer the days the better the leaves biomass and artemisinin content. *A. annua* grown in savanna region had the longest time to flowering than those planted in Lucknow (26º51' N) that flower earlier with life span of 75 days [SINGH & al. 1988]. This finding totally disagreed with MARCHESE & al. (2005) finding who proposed that *A. annua* is not suited to African continent because of short photoperiod that will make it flower early. Report shows that fertilizer types had significant effect on flowering time. The longest flowering time was observed in treatment with cow manure compost with medium proportion of 1:4, which is 77 days, while treatment with horse manure as a medium treatment showed the fastest flowering time of 42 days [PAMBUDI & al. 2017].

Effect of soil types on plant height at flowering

Loamy soil significantly (P<0.05) enhanced plant height parameter at the flowering stage of *A. annua* than those grown in sandy and clay soils (Table 3). The difference between the soils at this stage (plant height at flowering) may be ascribed to slightly alkalinity of the soil (7.8) (Table 5). This finding disagreed with OMER & al. (2013) that revealed that at flowering and maturing stage the differences in plant height between clay loamy and sandy loamy were insignificant (between 180 and 210 days after planting).

Effect of soil types on stem diametre of *A. annua*

Plants cultivated in clay soil showed a positive increase in the stem diameter than those cultivated in sandy and loamy soils. This may be attributed to present of high organic carbon, organic manure, Nitrogen, Phosphorus, Potassium, Calcium, Sodium and cation exchange capacity content than those found in loamy and sandy soils (Table 4). This goes in line with previous report that diameter of *A. annua* cultivated in clay loamy soil was higher than those cultivated in sandy loamy soil and it was true in the two seasons in all plant stages [OMER & al. 2013].

Table 3. Effect of soil types on days to flowering, plant height at flowering and stem diameter of *A. annua*

Treatment	Days to flowering	Plant height at flowering (cm)	Stem diametre (cm)
Sandy soil	150.75 ± 13.60	97.33 ± 7.57[b]	0.767 ± 0.611
Clay soil	147.50 ± 4.04	88.67± 1.528[b]	0.827 ± 0.433
Loamy soil	145.33 ± 3.79	118.00 ± 9.54[a]	0.593 ± 0.222
S.E	6.79	5.79	0.67

Replication × 5. Means followed by same superscript in a column are not significantly different (p>00.5). SE Standard error.

Table 4. Physical and chemical properties of soils used in the experiment

Treatment	pH	Org. C (%)	Org. M (%)	N (%)	P (mg/kg)	Ca	Mg	K (cmol kg$^-$)	Na	C.E.C.	% sand	% silt	% clay
Clay	7.0	0.74	1.28	0.116	0.88	0.85	0.70	0.85	0.65	8.6	38.4	17.1	44.5
Loamy	6.8	0.12	0.21	0.070	0.79	0.65	0.45	0.72	0.39	7.8	79.8	10.1	10.1
Sandy	7.6	0.38	0.66	0.039	0.65	0.50	0.25	0.23	0.22	5.6	89.60	6.59	3.81

pH: degree of acidity or alkalinity of a substances, Organic carbon (Org. C), Organic Mineral (Org. M), Nitrogen (N), Phosporus (P), Calcium (Ca), Magnesium (Mg), Potasium (K), Sodium (Na), Cation Exchange Capacity (C.E.C.).

Effect of soil type on artemisinin content in Artemisia *annua*

The results of HPLC analysis of artemisinin content of *A. annua* are presented in Table 5. The result revealed that sandy soil significantly (P≤0.05) had the highest artemisinin content in *A. annua* than clay and loamy soils. This is follow by clay and loamy soils with 17.9 and 15.70 µg/ml respectively. This difference may be attributed to slightly alkalinity of soil ph (7.6), fertility stress and the poor water retention property (Table 4). Similar report were made by OMER & al. (2013), that, *A. annua* plants growing in the sand loamy soil, showed a positive increase in artemisinin percentage comparing to those growing in clay soil. This result confirmed the previous works of GUPTA & al. (2002) and KUMAR & al. (2004). JESSING & al. (2013) revealed that, the largest contributor of artemisinin lost in *A. annua* was allelophatic effect of dead leaves in the soil environment. PRASAD & al. (1998) reported that, artemisinin content in the vegetative tissue was not influenced with a salinity stress of 10.4 dS/m. Variations in artemisinin content were reported in different plant parts, different stages of vegetative growth and strain origins [LAUGHLIN & al. 2002]. *A. annua* from Italy was reported to contain only 0.04% to 0.05% artemisinin dry weight. Artemisinin contents from other European origins ranged from 0.03% to 0.22% [CHARLES *&* al. 1991], while those obtained from China varied from 0.01% to 0.50% dry weight [KLAYMAN, 1985]. BUI THI & al. (2011) concluded that, the growth of the *A. annua* and the variations in artemisinin contents were attributed more strongly to environmental factors than to genetic factors because two similar clones planted at the two different study sites gives different artemisinin content.

Genotype and the environment are two factors that alter artemisinin content in *A. annua.* Artemisininvaries between 0.01 to 0.4% and some clones produce over 1% [DELABAYS & al. 1993]. Estimate of yield per hectare varies significantly, but reports indicate a yield of 10-15 kg/ha from well-managed plantations in Africa [WRIGHT, 2002]. Reports on the distribution of artemisinin and its derivatives throughout the plant is inconsistent. Artemisinin has been reported to be higher at the top of the plant in some clones [CHARLES & al. 1991] and equally distributed in others [LAUGHLIN, 1995].

Table 5. Artemisinin content of *A. annua* grown on different soils in Sokoto (µg/ml)

Soil type	**Ret time** (min)	**Area** (mAU*s)	**Conc.** (µg/ml)
Sandy	2.518 ± 0.007	22408 ± 4524[a]	37.73[a]
Loamy	2.498 ± 0.018	9322 ± 2872[b]	15.70[b]
Clay	2.494 ± 0.012	10616 ± 1289[b]	17.90[b]

means followed by same superscript in a column are not significantly different (p>00.5).

Conclusion

This study concludes that *A. annua* seeds could germinate and grow on different soil types in Sokoto geo-ecological environment andprobably all the surrounding areas that shares the same environmental, ecological and geographical factors for the first time as a promising cultivation area for *Artemisia annua* with artemisinin content. This new promising cultivation area may be used in a large scale in order to improve the overall supply of artemisinin. Soil types have no significant effect on germination, plant height, number of leaves, days to flowering and stem diametre. However, loamy soil significantly enhances plant height at flowering stage and length of the longest branch of *A. annua*. Artemisinin content of *A. annua* grown in sandy soil was significantly higher than that produced by clay and loamy soils. Pre-treatment of *A. annua* stem cuttings with IBA 400 and 600 ppm significantly increased percentage survival and reduce mean regenation time when compared with control. The overall growth performance parameters indicated that 600 ppm on apical stem cuttings were significantly superior to all other stem cuttings in term of percentage survival and mean regeneration time.

Recommendations

This study recommends the use of Sandy soil in cultivation of *A. annua* plant in savanna region of the country, for its influence artemisinin content accumulation to the farmers and pharmaceutical companies.

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ANALYSIS OF EFFECTS OF LEAD AND IRON TREATMENT ON EARLY SEEDLING GROWTH OF *ALBIZIA LEBBECK* L. (BENTH.) AND *EUCALYPTUS GLOBULUS* LABILL. *IN VITRO* STUDIES

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Abstract: Pollution by heavy metals in the environment is a worldwide problem. The aim of the research study was to record the effect of lead (Pb) and iron (Fe) elements on early seedling growth of *Albizia lebbeck* L. (Benth.) and *Eucalyptus globulus* Labill. The obtained results showed that higher level of lead (Pb) and iron (Fe) elements treatment present in the substrate had wide a spectrum of toxicity activity against seedlings growth performance of *A. lebbeck* and *E. globulus* as compared to control in lab conditions. Statistically analyzed data showed that seedling growth of *A. lebbeck* and *E. globulus* were reduced significantly ($p<0.05$) with increased concentrations of Pb and Fe 5 to 20 ppm as compared control (0 ppm). The percentage of seedling tolerance index of *A. lebbeck* and *E. globulus* showed different level of iron and lead. *A. lebbeck* showed greater tolerance indices (61.19%) in the presence of Fe than Pb treatments (50.39%). It was also noted that tolerance indices of *E. globulus* was reduced more in Fe (41.19%) as compared to Pb treatments (55.32%).

Keywords: Aromatic tree, heavy metals, seedling growth, tolerance, toxicity, tree.

Introduction

Heavy metals have a density of greater than 5 g/cm^3 [STOBRAWA & LORENC-PLUCINSKA, 2008]. The total amount and complexity of toxic pollutants in the environment are increasing day by day due to discharge of untreated chemicals from automobile and industries are a serious threat to the growth and quality of plant life [IQBAL & al. 2001; MEHBOOB & al. 2018; SHAFIQ & IQBAL, 2012; SHAFIQ & al. 2019]. The response of plant growth and metabolism to heavy metals has become the subject of great interest in recent years by plant ecologist. Among the heavy metals, lead and iron at higher levels are toxic element for germination and growth of plants. Soil pollution by heavy metals is a global environmental problem as has been affected about 235 million hectares land worldwide and on soil microbial properties [CHANDER & al. 2001; BERMUDEZ & al. 2012].

Lead (Pb^{2+}) is a wide spread dangerous heavy metal and strongly depends on its chemical speciation and toxicological potential [DRIBBEN & al. 2011; YUAN & al. 2011; SHAHID & al. 2012]. An inhibition in seedling growth of *Vigna radiata* (L.) Wilczek in the presence of 1.0 mM lead acetate was recorded [SINGH & al. 2003]. Pb level of 200 µM posed adverse effects on root morphological organization and root activity of *Elsholtzia argyi* H. Lév. plants [ISLAM & al. 2007]. The physiological responses and tolerance mechanisms to Pb stress in concentration of 50, 150, 300, 600, 800 and 1000 mg/L) for the *Salsola passerina* Bunge

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exhibited higher Pb tolerance in terms of the seed germination rate and bioactivities [HU & al. 2012]. Lead treatment at 05, 10, 15, 20 and 25 µmol L^{-1} declined seed germination, root, shoot and seedling length, seedling dry biomass and plant circumference of *Thespesia populnea* (L.) Soland. ex Corréa [KABIR & al. 2011]. The effect of different concentrations (0, 10, 20 and 30 ppm) of lead resulted reduction in root growth of two varieties of *Zea mays* L. [GHANI & al. 2010] commonly referred as maize.

Fe is essential element and moves to the seeds, most likely via the phloem, as the flow of the xylem is driven by transpiration [GRUSAK, 1994; YONEYAMA & al. 2015]. Fe is considered highly reactive and toxic via the Fenton reaction [MORRISSEY & GUERINOT, 2009]. $Fe^{(2+)}$ play a key role of in epithiospecifier protein activity [WILLIAMS & al. 2010].

Iron (Fe) is a metallic element and essential for plant growth. When Fe gets absorbed it binds to cell wall and other macromolecules in the cell. High concentration of Fe is found in roots, leaves and in stems [BHATTI & IQBAL, 1988]. Iron treatment significantly ($p<0.05$) reduced growth of plants [MORZECK & FUNICELLI, 1982; AL-HELAL, 1995; SHAFIQ & IQBAL, 2006]. Reduction in seed germination of all selected plants provided confirmation that Fe if available in excess is accountable in producing harmful effects. The work of VANGE & al. (2004) showed that higher concentrations of metals put adverse impacts on the growth of plants as seed germination is the most vulnerable stages in the development of plants. OZTURK & al. (2003) also observed that deceased in seedling fresh and dry weight in the presence of Fe treatment. Physiological effect on thirty three old days *Arabidopsis thaliana* seedlings to the interaction of iron deficiency and nitrogen form recorded [KARRAY-BOURAOUI & al. 2010].

Albizia lebbeck and *Eucalyptus globulus* are multipurpose economic value trees from which wood is used for both the industrial and medicinal purposes. *A. lebbeck* and *E. globulus* is widely used as raw material for the manufacturing of paper, timber, and packaging material, aromatic and in oil medicines industry in Pakistan. This study aims to evaluate the toxic effects of lead (Pb) and iron (Fe) salt on seed germination and early seedling growth performance of *A. lebbeck* and *E. globulus* under laboratory conditions.

Materials and methods

The different concentration of lead (Pb) and iron (Fe) salt in lead nitrate ($PbNO_3$) and ferrous sulphate ($FeSO_4$) for metal treatment were prepared. Stock solutions were prepared by weighting the 1.29 g and 2.71 g of $PbNO_3$ and $FeSO_4$ respectively and put them into two separate volumetric flask (1000 ml) and filled with distilled water up to mark on volumetric flask. To prepare the 5, 10, 15 and 20 ppm concentrations of both metals (Pb and Fe) 0.5, 1.0, 1.5 and 2.0 ml was sucked respectively from both stock solutions and added into volumetric flask (100 ml) and filled with distilled water up to the mark on flask. The certified seeds of *A. lebbeck* and *E. globulus* were collected from National seed store of Bhakkar. The seeds were surface sterilized with 2% of sodium hypochlorite (NaOCl) solution for two minutes to prevent fungal infection after that all the sterilized seeds were thoroughly washed with distilled water. Because of hard seeds coat pretreatments of seeds by soaking them in fresh water for 15 hours was carried out. The top trimmings of seeds were cut with the help of hygienic scissors to reduce any achievable seed dormancy. This mechanical scarification is a best treatment for first rate seed germination as well as best progression to break hard seed coat. The Petri dishes were washed to drop off the chances of further fungal infectivity. The Petri dishes and filter papers were sterilized in autoclaved. Afterward the five seeds on the filter paper (Whatman No. 42) were placed in medium size Petri dishes (90 mm) and had two replicates for each treatment of metals. Initially,

3 ml of distilled water was given to control group and 3 ml of each concentration of both metal solutions were given to each set of respective treatment. In a while, on daily basis the old solution was removed by sucking from Petri dishes in order to reduce the chances of seed turgidity and later on 2 ml of fresh solution of each metal concentration was added to each set of respective treatment. The experiment was kept in laboratory conditions at room temperature (38 ± 4 °C) and experiment was lasted for 15 days. The experiment was completely randomized and replicated twice. The germination was scored as protrusion of the radical through the testa. The seed germination percentage, root, shoot and seedling length of *A. lebbeck* and *E. globulus* were measured. Seedling dry weight was obtained after drying the samples in an oven at 80 °C for 24 hours.

Tolerance index (T.I.) was determined using the following formula given by IQBAL & RAHMATI (1992):

T.I. = (Mean root length in metal solution / Mean root length in distilled water) x 100

Statistical analysis

Data of different growth parameters were analyzed statistically (SPSS 20) on personal computer by analysis of variance and Duncan Multiple Range Test at $p<0.05$ level.

Results and discussion

High concentrations of metals in the environment can create harmful effects on germination and growth of plants. In present time most heavy metals especially lead (Pb) and iron (Fe) became important due to their regular increase in the atmosphere. Most of the plant species under applied stress conditions are probably to be badly influenced by heavy metals. The influence of heavy metals on vegetation depends upon the quantity of that toxic metal taken by the plant [HAILING & al. 1991]. Sensitivity of plants and toxicity of heavy metals are affected by the concentration of given metal, length of exposure period and mechanism of biological processes occurring in the plant species [ERNST & al. 1992].

In present study the seedling growth of *A. lebbeck* and *E. globulus* was carried out in different concentrations of lead and iron under laboratory conditions (Table 1-4, Figure 1). Statistically analyzed data showed that seed germination, seedling growth and tolerance indices of seedlings were reduced significantly ($p<0.05$) with increased concentrations of both applied metals (Pb and Fe) as compared control. Results presented in Table 1-2 revealed that lead treatment at 05 ppm application created significant ($p<0.05$) results on seedling length (9.35 cm) of *A. lebbeck* as compared to control (13.04 cm). The root length (2.74 cm) and shoot length (5.74 cm) of *A. lebbeck* showed further $p<0.05$) reduction with lead treatment at 10 ppm. Pb toxicity found responsible for causing a detrimental effect on seedling growth *Leucaena leucocephala* (Lam.) de Wit small fast growing [IQBAL & SHAFIQ, 1998]. KOPITTKE & al. (2007) found a concentration as low as 1 μM lead (Pb) highly toxic to plants. In an experiment, the relative fresh mass of cowpea (*Vigna unguiculata* L. Walp.) was reported reduced by 10% at a Pb^{2+} activity of 0.2 μM for the shoots and at a Pb^{2+} activity of 0.06 μM for the roots. The primary site of Pb^{2+} toxicity was the root, causing severe reductions in root growth, loss of apical dominance, the formation of localized swellings behind the root tips and the bending of some root tips of *V. unguiculata*. Pb was found to accumulate primarily within the cell walls and intercellular spaces. Maximum suppression of root length (1.71 cm), shoot length (4.07 cm) and seedling length (5.78 cm) were recorded at highest concentrations of Pb (20 ppm). Results of selected plants species showed that seedling growth, seedling weight (fresh and dry) of *A. lebbeck* were reduced in all treatments (05, 10, 15 and 20 ppm) of Pb as compared to control (0

ppm). Lead treatment at 10 and 15 ppm progressively decreased seedling dry weight 0.34 and 0.29 g as compared to control (0.46 g) of *A. lebbeck*. The assessment of seedling dry weight of *A. lebbeck* followed the same reduction array as observed for seedling fresh weight in different applications of lead.

Table 1. Effects of Lead (Pb) on root, shoot, seedling growth and seedling fresh and dry weight of *Albizia lebbeck*

Treatment Lead (Pb) ppm	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Seedling fresh Weight (g)	Seedling dry Weight (g)
00	5.47 ± 0.13a	7.57 ± 0.49a	13.04 ± 0.52a	0.70 ± 0.005a	0.46 ± 0.005a
05	3.54 ± 0.47b	5.81 ± 0.27b	9.35 ± 0.35b	0.50 ± 0.007b	0.37 ± 0.005ab
10	2.74 ± .35bc	5.74 ± 0.53bc	8.48 ± 0.70bc	0.46 ± 0.009bc	0.34 ± 0.008b
15	2.24 ± 0.09c	5.24 ± 0.21bc	7.48 ± 0.26c	0.40 ± 0.004bc	0.29 ± 0.006bc
20	1.71 ± 0.27cd	4.07 ± 0.56c	5.78 ± 0.84d	0.36 ± 0.003c	0.26± 0.005c

Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error

The seedling length (root, shoot), seedling weight (fresh and dry) and tolerance indices of *A. lebbeck* were decreased with the application of iron (Fe) at 05, 10, 15 and 20 ppm concentrations as compared to control (0 ppm). With increasing 05, 10, 15 and 20 ppm concentrations of iron treatment also markedly reduced the root, shoot, seedling length, seedling fresh and seedling dry weight of *A. lebbeck* (Table 2). Chemical compounds exhibit toxicity via many mechanisms of toxic action [REN, 2003]. Some of morphological parameters, root, shoot and seedling length of *A. lebbeck* got significant ($p<0.05$) reduction at varying concentrations of Fe treatment than control. Maximum suppression of root and shoot growth of *A. lebbeck* with Fe (20 ppm) treatment with relation to root length (2.31 cm), shoot length (5.17 cm) and seedling length (7.48 cm) were recorded. Plant responses to metals can be considered as dose dependent. Toxicity appears to be the results of several interactions. While increased iron treatments 5, 10, 15 and 20 ppm reduced the fresh weight from 0.68, 0.65, 0.56 and 0.48 g as related to control (0.70 g) of *A. lebbeck*. The assessment of seedling dry weight of *A. lebbeck* followed the same reduction array as observed for seedling fresh weight in different applications of iron. Heavy metal, cadmium inhibited biomass production as well as the absorption of K, Ca, Mg, Fe and dramatically increased Cd accumulation in both roots and shoots of *Linum usitatissimum* L. [BELKHADI & al. 2010].

Table 2. Effects of Iron (Fe) on root, shoot, seedling length, seedling fresh and dry weight of *A. lebbeck*

Treatment Iron (Fe) ppm	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Seedling fresh Weight (g)	Seedling dry Weight (g)
00	5.47 ± 0.13a	7.58 ± 0.49a	13.05 ± 0.52a	0.70 ± 0.005a	0.46 ± 0.005ab
05	4.07 ± 0.33b	6.79 ± 0.09ab	10.86 ± 0.31b	0.68 ±0.003ab	0.52 ± 0.003a
10	3.34 ± 0.24bc	6.44± 0.19b	9.78 ± 0.43c	0.65 ±0.009ab	0.45 ± 0.004ab
15	2.34 ± 0.09c	5.22 ± 0.16c	7.56 ± 0.25d	0.56 ± 0.008b	0.41 ± 0.009b
20	2.31± 0.07cd	5.17 ± 0.16cd	7.48 ± 0.20de	0.48 ±0.001bc	0.33 ± 0.002c

Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error

Lead (05, 10, 15 and 20 ppm) application responded variably with seedling growth performances parameters of *E. globulus* than control (Table 3). Lead concentrations at 20 ppm created significant ($p<0.05$) effects on length of root (0.91 cm), shoot (1.03 cm) and seedling length (1.94 cm) of *E. globulus.* Seedling fresh weight of *E. globulus* was highest in control seedlings (0.0345 g) and gradually declined with the increase in Pb concentration from 05 to 20 ppm. The decrease in seedling fresh weight 0.0320, 0.0311 and 0.0231 g of *E. globulus* was recorded for those seedlings which were treated with 05, 10 and 15 ppm concentrations of Fe. The lowest seedling fresh weight (0.0178 g) was recorded for those seedlings which were treated with 20 ppm concentrations Pb. Similarly, the seedling dry weight of *E. globulus* was also reduced 0.244, 0.0216 and 0.0132 g in Pb applications at 05, 10 and 15 ppm as associated to control (0.0269 g). It reduced spontaneously with increasing Pb concentration in substrate. The lowest seedling dry weight (0.0100 g) was recorded for those seedlings of *E. globulus* which were treated with 20 ppm concentrations Pb.

Table 3. Effects of lead (Pb) on seed germination, seeding growth and seedling dry weight of *Eucalyptus globulus*

Treatment lead (Pb) ppm	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Seedling fresh weight (g)	Seedling dry weight (g)
00	1.22 ± 0.04a	2.44 ± 0.02a	3.66 ± 0.21a	0.0345 ± .003a	0.0269 ± 0.003a
05	1.16 ±0.03ab	2.43 ± 0.08a	3.59 ± 0.09ab	0.0320 ± .003ab	0.0244 ± 0.002b
10	1.09 ± 0.02b	2.19 ± 0.09b	3.28 ± 0.11b	0.0311 ± .003ab	0.0216 .003bc
15	0.97 ± 0.03c	1.32 ± 0.15c	2.29 ± 0.15c	0.0231 ±0.002b	0.0132 ±0.001d
20	0.91 ± .03cd	1.03 ± 0.10d	1.94 ± 0.17cd	0.0178 ±0.003c	0.0100 ±0.003e

Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Err

Similarly, iron (05, 10, 15 and 20 ppm) application variably reduced the selected seedling growth performances parameters of *E. globulus* than control (Table 4). An increase in the concentrations of iron up at 20 ppm created significant ($p<0.05$) effects on length of root (0.79 cm), shoot (1.00 cm) and seedling length (1.79 cm) of *E. globulus*. Seedling fresh weight of *E. globulus* was highest in control seedlings (0.0348 g) and gradually declined with the increase in Fe concentration from 05 to 20 ppm. The decrease in seedling fresh weight 0.0307, 0.0291 and 0.0286 g of *E. globulus* was recorded for those seedlings which were treated with 05, 10 and 15 ppm concentrations of Fe. Similarly, the seedling dry weight of *E. globulus* was also reduced 0.216, 0.0193 and 0.0126 g in Fe applications at 05, 10 and 15 ppm as associated to control (0.0269 g). The maximum decrease in seedling fresh weight (0.0166 g) was recorded for those seedlings which were treated with 20 ppm concentrations Fe. Seedling dry weight of *E. globulus* was also reduced (0.0126 g) in Fe applications at 15 ppm as associated to control (0.0269 g). It reduced spontaneously with increasing Fe concentration in substrate. The lowest seedling dry weight (0.0100 g) was recorded for those seedlings of *E. globulus* which were treated with 20 ppm concentrations Fe. ALIKAMANOGLU & al. (2011) reported he toxic effect of iron on growth factors, biochemical parameters, seedling fresh and dry weight and accumulation of trace elements in soybean plants (*Glycine max* L. Merrill).

A lot of evaluations have been made by scientist to access the impact of heavy metals on tolerance of plants. The tolerance of some plants to heavy metals is a precious ecological adaptation for their survival in a specific environment. The accumulation of heavy metals in soil influences on plant growth and ecosystem balance and there is a need to explore the mechanism of plant tolerance to heavy metals [GONG & al. 2019].

Table 4. Effects of Iron (Fe) on different growth parameters of *Eucalyptus globulus*

Treatment Iron (Fe) ppm	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Seedling fresh Weight (g)	Seedling dry Weight (g)
00	1.23 ± 0.03a	2.45 ± 0.02a	3.68 ± 0.53a	0.0348 ± 0.005a	0.0269 ± 0.003a
05	1.13 ± 0.03b	2.41 ± 0.09a	3.54 ± 0.23ab	0.0307 ± 0.009b	0.0216 ± 0.003b
10	1.02 ± 0.03c	1.90 ± 0.08b	2.92 ± 0.52b	0.0291 ± 0.003bc	0.0193 ± 0.003bc
15	0.90 ± 0.03d	1.12± 0.15c	2.02 ± 0.15bc	0.0286 ± 0.004c	0.0126 ± 0.003c
20	0.79 ± 0.03e	1.00 ± 0.10cd	1.79 ± 0.19c	0.0166± 0.005d	0.0100 ± 0.003cd

Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error

The tolerance of *E. globulus* to Pb and Fe gradually reduced with the increase in Pb and Fe concentration than control as presented in Figure 1. Lead at 05, 10, 15 and 20 ppm produced 95.08, 89.34, 79.51 and 74.59% tolerance of *E. globulus* respectively. The metal resistance is an unusual character found only in well adapted plant species. Iron treatment at similar concentrations produced 74.41, 61.06, 42.78 and 42.23% tolerance in *A. lebbeck*. It was determined that inhibitory effects of Pb were more rigorous on all growth variables of *A. lebbeck* as compared to Fe treatment. Similarly, KABIR & al. (2011) studied the tolerance of *Samanea saman* for Cu, Fe, Pb and Zn under laboratory conditions and showed that with increasing concentrations of metals reduced seed germination. Iron treatment at similar range of treatment produced 91.87, 82.93, 73.17 and 64.23% tolerance in *E. globulus* respectively. These results were similar with the results of KABIR & al. (2008).

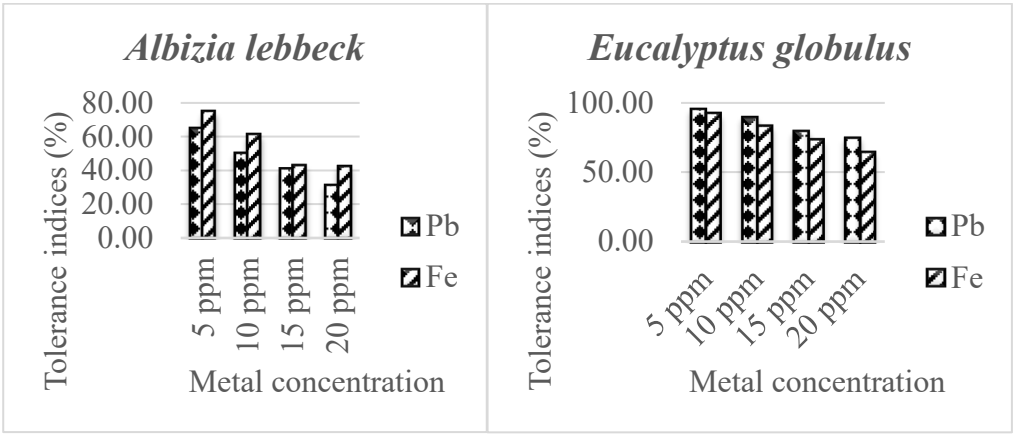

Figure 1. Tolerance indices (%) of *Eucalyptus globulus* and *Albizia lebbeck* in different concentration of Pb and Fe.

According to their results tolerance indices of *Thespesia populnea* progressively decreased with the increasing concentration of heavy metal. These results were also supported by the work of BOYD & al. (1994). It was cleared from results that Fe applications created more deadly effects on *E. globulus* seedlings than Pb applications at all concentrations. Lead is found more toxic to seedling growth of *A. lebbeck* as compared to Fe while *E. globulus* is more tolerant to Fe than Pb. The cause of low tolerance to Pb and Fe could be due to disturbance in physiological processes.

Conclusion

This study concludes that treatment of different concentration (5, 10, 15 and 20 ppm) lead and iron gradually deceased the rate of seed germination percentage, seedling growth and seedling dry weight of *A. lebbeck* and *E. globulus* as compared control (0 ppm) treatment. An increase in Pb and Fe level 5 to 20 ppm concentration also gradually decrease seedling tolerance index of *A. lebbeck* and *E. globulus* as compared to control treatment.

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THE IMPACT OF AUTOMOBILE POLLUTED SOIL ON SEEDLING GROWTH PERFORMANCE IN SOME HIGHER PLANTS

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Abstract: Rapid increase in automobile density and discharge of different types of pollutants from automobile are a serious issue for whole civilized world and in Bhakkar also.Vehicle emission from automobiles released an enormous quantity of toxic pollutants likewise nitrogen dioxide, sulfur dioxide, carbon mono oxide, heavy metals particularly lead, cadmium in environment and produced harmful effects on germination and growth of plants. This study was aim to investigate the effect of automobile polluted soil on the growth of some tree species. In present study the variation in seedling growth performance of three different selected tree species namely, *Acacia nilotica* L., *Albizia lebbeck* L. (Benth.) and *Eucalyptus globulus* Labill. raised in roadside polluted soils of District Bhakkar, Pakistan were recorded in the pots. Results showed that root, shoot, seedling length, number of leaves, and seedling dry weight of *Acacia nilotica* grown in polluted road soils of Bhakkar-Khansar road showed a significant ($p<0.05$) decline. Statistical analysis of recorded data showed that root growth and leaf area of *Albizia lebbeck* in the soil of Bhakkar-Khansar road soil significantly ($p<0.05$) decreased as compared to control. Similarly, a significant ($p<0.05$) reduction in shoot, seedling length, number of leaves, leaf area and seedling dry weight performance of *E. globulus* in polluted soils of Bhakkar-Notak was recorded.

Keywords: biomass, soil pollution, tree, vehicles emission.

Introduction

Vehicle passengers and goods transport from one place to another and they have made life easy and convenient. Motor vehicle traffic is a major source of air pollution in urban areas and contributing 57%-75% of total emissions [WHO, 2006]. The automobiles activities emit various compounds likewise, nitrogen, sulphur, hydrogen fluorides, hydrocarbons, particulate matter, peroxy acetyl nitrates and heavy metals into atmosphere which put harmful effects on human, animals and to the trees [UABOI-EGBENNI & al. 2009]. The use of diesel and petrol fuel in automobiles contributes various pollutants into air with different concentrations depending upon the operating conditions of automobiles [COLVILE & al. 2000]. In China, vehicles participate only 7.2% in 1995 but it would grow up to 11.3% in 2020 [STREETS & al. 2001]. It is estimated that annual increase of vehicles is 37% in Pakistan [ILYAS, 2007]. Water and carbon dioxide are produced in the complete combustion of petroleum and diesel but usually incomplete combustion occur giving rise to various solid particles, liquids and gases [ANDA & ILLES, 2012]. Different plant species vary in extent of response to vehicle pollutants exposure. Researchers are claiming that vehicle emission is responsible for increase the level of toxic pollutants in environment due to ever increase in number of automobiles [SULISTIJORINI &

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al. 2008; KABIR & al. 2012; SHAFIQ, 2002; SHAFIQ & IQBAL, 2012] and ultimately negatively affecting germination and growth of plants. ZHAO & al. (2009) accounted an unfavorable effects of air pollution on growth of plants that might be due to some poisonous substances releasing from automobiles. Effect of automobile polluted soil on early seedling growth performance and biomass production of Neem (*Azadirachta indica* A. Juss.) [PARVEEN & al. 2016].

Species description

Acacia nilotica (L.) Willd. ex Delile is synonym of *Vachellia nilotica* (L.) P. J. H. Hurter & Mabb. [WFO, 2022] and belongs to family Fabaceae and used as application of afforestation in forestry. It grows commonly 3-15 meters high or sometime low as 1.5 meters. The seed germinate after a period of warm moist condition after scarification [PARSONS & CUTHBERTSON, 1992]. Its wood is useful in the production of fuel wood, charcoal, paper and medicines industry [KANAK & SAHAI, 1994]. When this plant is young, bark is whitish but it changed to dark gray when it gets matures and has deep taproot system with branching surface lateral roots [COX, 1997; MACKEY, 1997]. The fruit of *Acacia nilotica* is leathery pod and the color of pod varies from brown to dark gray, straight to curved and glabrous or velvety [BROWN & CARTER, 1998]. Growth rates are variable, it may mature in nine months under good environmental conditions or not for up to 13 years under harsh conditions [KRITICOS & al. 1999]. *Acacia nilotica* helps to improve the rural economy by providing fodder, timber, fuel, gum and medicines. This tree also play role to increase the soil fertility under its canopy [PANDEY & al. 2000]. *Acacia nilotica* is used in bridges, railway sleepers, sports goods, building of boats, carts, carriages, and construction of doors, window frames, decorative cabinets and carpentry work [KUMAR & KUMUD, 2010]. The distribution of *Acacia nilotica* includes Africa, Indian subcontinents and also planted in Pakistan along the roadside as shade tree along the field boarder as shelterbelts and windbreaker. It is of great value on both national and international level for timber and decorative wood and aromatic oil.

Albizia lebbeck L. (Benth.) is a member of family Fabaceae and subfamily Mimosaceae. *Albizia lebbeck* is commonly called as Siris tree, Shrin and Vaagei. It is deciduous woody tree and cultivated in gardens as ornamental plants, along roadsides as shade tree, on irrigated plantation and in farmlands. This deciduous tree is found all over the world especially in Pakistan, India, Bangladesh tropical and subtropical Africa and Asia [AHMAD & BEG, 2001]. It is large multi stemmed tree with widespread canopy (30 m). *Albizia lebbeck* is used as fodder crop of high quality for animal food. Its tree has shallow and extensive root system making it helpful in soil conservation through soil erosion control [PRINSEN, 1986]. *Albizia lebbeck* is a valuable timber species also used for furniture, flooring, carving posts and in various kinds of agricultural implements. The bark contains 15% tannin used in tanning and dying industry. Due to property of high saponin contents also used in detergents [VARSHNEY & BADHWAR, 1970]. Its bark produces brown reddish gum used as a part of Arabic gum [FAROOQI & KAPOOR, 1968]. The seed oil is used in the treatment of lesions in leprosy disease [RAGUPATHY & MAHADEVAN, 1991].

Eucalyptus globulus Labill. is a tall tree and member of family Myrtaceae. Most of the species of *E. globulus* are tall trees with height of 100 meter and girth of 20 meter. Almost all species of it are evergreen and very few species are deciduous [POHJONEN, 1989]. *E. globulus* is tolerant to moisture stress and low soil fertility. *E. globulus* is planted in garden, along roadside and parks. It is also found useful for fuel wood, charcoal, timber, plywood, paper pulp, oil, fiberboard, tannin, shade and shelter, source of nectar for honey and ornamental purposes [MOGES, 1998].

The ever increase in vehicle density is producing environmental pollution issues and is affecting growth of roadside plants. There is no scientific study is available on the effect of automobile polluted soil of Bhakkar on plant growth. Keeping in view of the constant increase in traffic activities which is polluting the soil of the area, thecurrent research experiments was conducted with the aim to compare the effects of automobile polluted soil on three different economic importanttree species namely, *Acacia nilotica* L. Willd. ex Delile, *Albizia lebbeck* L. (Benth.) and *Eucalyptus globulus* Labill. of Pakistan.

Material and methods

Description of experimental site

Bhakkar, is the principal city of Bhakkar District and located in Punjab, Pakistan. It lies on the left bank of the Indus river. It stands on the edge of the Thal or sandy plain overlooking the low-lying alluvial lands along; the river, a channel of which is navigable as far as Bhakkar during the floods. To the west of the town the land is low, well cultivated, and subject to inundation, while to the east the country is high and dry, treeless, and sandy. A rich extent of land irrigated from wells lies below the town, protected by embankments from inundations of the Indus, and produces two or three crops in the year.

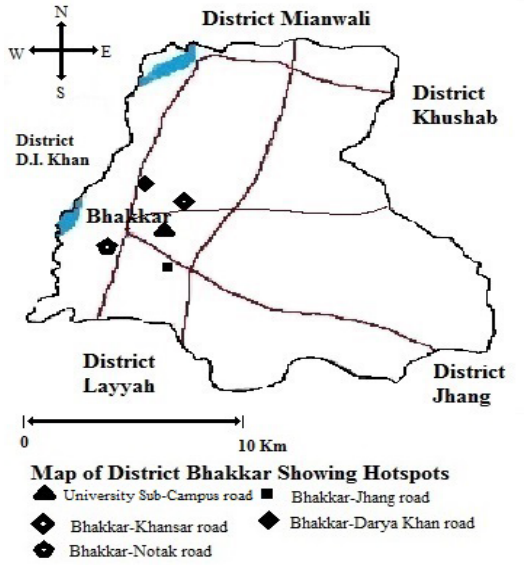

Figure 1. Map of District Bhakkar showing selected roads

Soil samples were collected from five different roadsides sites namely, (A = University Sub-Campus road, B = Bhakkar-Darya Khan road, C = Bhakkar-Jhang road, D = Bhakkar-Notak road and E = Bhakkar-Khansar road) at the depth of 0-45 cm from every site during 2018 and climatic data (Figure 1). The composit soil samples were collected from each site at equal distance. The soil samples were taken to laboratory in polythene bag and kept at room temperature for drying. All collected soil samples were slightly crushed and passed through a 2 mm sieve to get equal size particle distribution. The air dried soil was then shifted into clean

polythene bags, labeled and stored in the laboratory. Weekly climatic data of District Bhakkar during growth experiments (01-06-2018 to 31-07-2018) was recorded (Table 1).

Table 1. Weekly climatic data of District Bhakkar during growth experiments (01-06-2018 to 31-07-2018)

Weeks	Temperature (°C)			Relative Humidity (%)			Sun shine (hours)	Weather outlook
	Min.	Max.	Mean	Min.	Max.	Mean		
1	38	44	40	66	74	70	14:15	Partially cloudy
2	39	43	41	56	72	64	14:25	Fair & Hot
3	35	43	39	58	72	65	14: 32	Warm & Humid
4	37	49	43	66	80	73	14: 25	Hot & Dry
5	40	44	42	61	85	73	14: 18	Hot & Dry
6	44	42	38	64	80	72	14: 13	Warm & Humid
7	37	43	40	56	92	74	14: 02	Hot & Dry
8	33	45	39	56	80	68	13: 56	Hot & Dry

Abbreviations used: Min. = Minimum; Max. = Maximum.
Source: Main Line Lower Land Reclamation Research Station Chak No. 37 TDA, Bhakkar.

The experiment for influence of polluted soil collected from five different roadsides sites (namely A = University Sub-Campus road, B = Bhakkar-Darya Khan road, C = Bhakkar-Jhang road, D = Bhakkar-Notak road and E = Bhakkar-Khansar road) on seedling growth of *Acacia nilotica* L. Willd. ex Delile, *Albizia lebbeck* L. (Benth.) and *Eucalyptus globulus* Labill. was conducted at the Department of Biological Sciences, University of Sargodha, Sub-Campus Bhakkar (Punjab, Pakistan) under natural environmental condition in pot. The vigorous, healthy and same size seeds of *Acacia nilotica*, *Albizia lebbeck* and *E. globulus* were collected from local National seed store of Bhakkar. The seeds were surface sterilized with 0.20% of sodium hypochlorite (NaOCl) solution for two minutes to avoid any fungal contamination and washed with thoroughly with distilled water.

The micropyle top of seeds of these plants' species were marginally cut to some extent with hygienic scissors to break external seed dormancy. Ten seeds were sown at 1.00 cm depth in earthen pots containing the soil of different polluted road such as A = University Sub-Campus road as control, B = Bhakkar-Darya Khan road, C = Bhakkar-Jhang road, D = Bhakkar-Notak road and E = Bhakkar-Khansar road. The earthen pots watered regularly. After two weeks of seed germination, equal size of seedlings was transplanted in plastic pots of 9.8 cm in depth and 7.00 cm in diameter. There were three replicates of each plant species seedling for each polluted roadside soil. One seedling was transplanted in each plastic pot and seedlings were watered regularly. After every week pots reshuffling were also carried out to prevent light shade or any other environmental effect. At the completion of experiment (eight weeks) the seedlings were removed from plastic pots, washed their roots with fresh water and measured the root, shoot, seedling length and leaf area with the help of iron scale and number of leaves were also counted. Seedling fresh weight was determined with the help of electrical balance. After that the seedlings were dried in a thermostatic drying oven at 80 °C and then oven dried weight of leaves, root, shoot and seedling were also determined by using electrical balance.

The root shoot ratio, leaf weight ratio, leaf area, specific leaf area and leaf area ratio were also determined by formula as given by ATIQ-UR-REHMAN & IQBAL (2009).

$$\text{Root/Shoot ratio} = \frac{\text{Root dry weight}}{\text{Shoot dry weight}}$$

$$\text{Leaf weight ratio} = \frac{\text{Leaf dry weight}}{\text{Total plant dry weight}}$$

$$\text{Leaf area} = \text{Leaf length} \times \text{Leaf width} \times \frac{2}{3}$$

$$\text{Spacific leaf area} = \frac{\text{Leaf area}}{\text{Leaf dry weight}}$$

$$\text{Leaf area ratio} = \frac{\text{Leaf area}}{\text{Total plant dry weight}}$$

Statistical analysis

Data of different growth parameters were analyzed statistically by analysis of variance (ANOVA) and Duncan Multiple Range Test (Duncan, 1995) at $p<0.05$ level on personal computer.

Results and discussion

The transport sector is an important source of environmental pollution. The chaotic and rapid vehicle growth is producing massive environmental pollution issues and is affecting not only the growth of plants but also might be influencing on the different characteristics of soil of the area. The influence of polluted soil collected from five different roadsides sites (namely A = University Sub-Campus road, B = Bhakkar-Darya Khan road, C = Bhakkar-Jhang road, D = Bhakkar-Notak road and E = Bhakkar-Khansar road) on seedling growth and seedling dry weight of *Acacia nilotica* (L.) Willd. ex Delile, *Albizia lebbeck* L. (Benth.) and *Eucalyptus globulus* Labill. with some variation was recorded (Table 2-10). Statistical analysis of recorded data showed that root, shoot, seedling length and number of leaves of *Acacia nilotica* were significantly ($p<0.05$) reduced in soil of Bhakkar-Khansar road as compared to other soil treatment (Table 2).

Table 2. Growth of *Acacia nilotica* in soil of different polluted roads

Roads	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Number of leaves	Leaf area (cm^2)
A	13.31± 0.24b	30.29 ± 0.95a	43.60 ± 1.16b	8.33 ± 0.15ab	7.46 ± 0.22a
B	10.30 ± 0.28bc	26.53 ± 0.77b	36.83 ± 1.05c	7.43 ± 0.44b	6.07 ± 0.30b
C	18.27 ± 0.36a	28.63 ± 1.22ab	46.90 ± 1.56a	10.00 ± 0.65a	6.72 ± 0.25ab
D	11.53 ± 0.34bc	23.57 ± 0.85c	35.10 ± 1.18c	6.55 ± 0.23bc	3.50 ± 0.23cd
E	8.44 ± 0.21c	20.67 ± 0.52d	29.11 ± 0.69d	5.81 ± 0.17bc	3.73 ± 0.25c

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. ± Standard Error. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level.

The significant reduction in seedling growth of *Acacia nilotica* wasconsideredmainly depended upon pollutants released from automobiles. Air pollution directly affects plants via leaves or indirectly via soil acidification [LIU & DING, 2008]. Root length, seedling length and number of leaves and seedling dry weight of *Acacia nilotica* grown in Bhakkar-Jhang road soil was recorded significantly greater as compared to University Sub-Campus road, Bhakkar-Darya

Khan road, Bhakkar-Notak road and Bhakkar-Khansar road showed some degree of tolerance to soil pollution (Table 3).

Table 3. Effects of soil of different polluted roads on root, shoot, leaf and seedling dry weight of *Acacia nilotica*

Roads	Root dry weight (g)	Shoot dry weight (g)	Leaf dry weight (g)	Seedling dry weight (g)
A	0.07 ± 0.002ab	0.13 ± 0.002ab	0.013 ± 0.002a	0.20 ± 0.001ab
B	0.07 ± 0.002ab	0.12 ± 0.001ab	0.009 ± 0.002ab	0.19 ± 0.001ab
C	0.08 ± 0.002a	0.15 ± 0.002a	0.010 ± 0.002ab	0.23 ± 0.002a
D	0.05 ± 0.001b	0.09 ± 0.001b	0.007 ± 0.001b	0.14 ± 0.001b
E	0.04 ± 0.001bc	0.09 ± 0.001b	0.008 ± 0.001b	0.13 ± 0.002b

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. ± Standard Error. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level.

Seedling fresh weight was significantly ($p<0.05$) high in plants developed from the soil of Bhakkar-Jhang road (0.32 g) as compared to control while other three polluted roads showed significant ($p<0.05$) reduction with control. Maximum seedling dry weight was recorded as 0.23 g for Bhakkar-Jhang road soil which was significantly ($p<0.05$) decreased to 0.20 g for control soil. The seedling's fresh weight and dry weight of *Acacia nilotica* showed significant ($p<0.05$) variations in different polluted roadside soils. Reduction in biomass of *Acacia nilotica* may be due to imbalance in carbon dioxide exchange as a result of which photosynthesis activities got reduced [SHAFIQ, 2002]. Only Bhakkar-Darya Khan road soil showed nonsignificant result with control. Specific leaf area of *Acacia nilotica* raised in Bhakkar-Jhang road soil demonstrated significant ($p<0.05$) increase in other polluted road side soil (Table 4). A better root/shoot ratio, leaf weight ratio and leaf area ratio of *Acacia nilotica* raised in Bhakkar-Khansar road soil was found as compared to other polluted road side soil.

Table 4. Root/shoot, leaf weight ratio, specific leaf area and leaf area ratio of *Acacia nilotica* in soil of different polluted roads

Roads	Root/Shoot ratio	Leaf weight ratio	Specific leaf area ($cm^2\ g^{-1}$)	Leaf area ratio ($cm^2\ g^{-1}$)
A	0.54 ± 0.02ab	0.07 ± 0.003a	573.85 ± 9.94b	37.30 ± 1.97a
B	0.58 ± 0.04a	0.05 ± 0.001b	674.44 ± 6.11a	31.95 ± 1.84b
C	0.53 ± 0.02ab	0.04 ± 0.001bc	672.00 ± 8.59a	29.22 ± 1.28bc
D	0.56 ± 0.03ab	0.05 ± 0.001b	500.00 ± 7.18c	25.00 ± 1.83c
E	0.44 ± 0.01b	0.06 ± 0.002ab	466.25 ± 5.97cd	28.69 ± 1.43bc

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. ± Standard Error. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level.

Plants growing along the roadsides facing continuously different challenges which would cause variations in the biochemical processes, total chlorophyll contents and storage of some metabolites [AGBAIRE & ESIEFARIENRHE, 2009]. Different plant species vary in extent of response to vehicle pollutants exposure. This variation in seedling growth of selected plant species may be related with the amount of vehicle pollutants [HONOUR & al. 2009]. Our results were according to the findings of IQBAL & SHAZIA (2004) that decrease in length (root, shoot and seedling) along with fresh and dry weight of *Albizia lebbeck* by the exposure

to different vehicle pollutants (Table 5-6). In another study seedling growth of *Albizia lebbeck* and *Pongamia pinnata* showed significant ($p<0.05$) reduction in root, shoot and seedling length raised from polluted road soils [QADIR & IQBAL, 1991]. From the results of present research work it was indicated that soil of study area might be disturbed in future due to emission and settling of toxic pollutant from vehicles.

The observations recorded in the present study clearly indicated that pollutants emitted from the automobile exhaust exercised a decisive influence on seedling growth of *Albizia lebbeck*. The significance of germination and seedling growth is an extensively recognized factor in plant growth. A significant variation in seedling growth of *Alibizia lebbeck* raised in different polluted roadside soils of District Bhakkar was recorded. Statistical analysis of recorded data showed that the polluted soil influenced root, shoot and seedling length. number of leaves and leaf area, root, shoot, leaves dry weight, seedling fresh and dry weight, root/shoot ratio, leaf weight ratio, specific leaf area and leaf area ratio of *Albizia lebbeck.* Statistical analysis of recorded data showed that seedling length and number of leaves of *Albizia lebbeck* in the soil of Bhakkar-Notak road soil. The shoot, seedling length and number of leaves of *Albizia lebbeck* were significantly ($p<0.05$) greater in the soil of Bhakkar-Jhang road (Table 5).

Table 5. Growth of *Albizia lebbeck* in soil of different polluted roads

Roads	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Number of leaves	Leaf area (cm^2)
A	9.51 ± 0.59b	17.19 ± 0.48c	26.70 ± 1.19c	8.23 ± 0.27b	8.76 ± 0.09a
B	12.32 ± 0.96a	21.81 ± 0.66b	34.13 ± 1.35b	9.43 ± 0.33ab	7.43 ± 0.14ab
C	10.62 ± 0.52ab	27.81 ± 0.65a	38.43 ± 0.95a	10.11 ± 0.05a	6.42 ± 0.05b
D	7.81 ± 0.32bc	10.71 ± 1.02d	18.52 ± 1.25e	6.19 ± 0.80c	5.39 ± 0.12bc
E	6.25 ± 0.38c	14.01 ± 0.72cd	20.26 ± 0.98d	7.07 ± 0.33bc	4.12 ± 0.07c

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error

In present study the seedling dry weight performances of *Albizia lebbeck* was responded differently when raised in different polluted roadside soils of District Bhakkar (Table 6). A significant reduction in seedling fresh and dry weight, root, shoot, leaves dry weight of *Albizia lebbeck* was recorded in the soil of Bhakkar-Notak road. Leaf weight ratio was significantly ($p<0.05$) high (0.07) in control soil. The seedling growth of *Albizia lebbeck* showed better growth in soil of Bhakkar-Jhang road and Bhakkar-Darya Khan road as compared to control and soils of other roads. All the growth variables were significantly ($p<0.05$) reduced in soils of Bhakkar-Notak road and Bhakkar-Khansar road indicating its less tolerance and adaptability to these polluted soils. The seedling length of different plant species exhibited the reduction in root and shoot length as these parts are exposed to either direct or indirect automobile pollutants present in the soil [ALLOWAY & AYRES, 1997]. Maximum value of specific leaf area was recorded in Bhakkar-Darya Khan road soil (674.44 cm^2g^{-1}) and Bhakkar-Jhang road soil (672.00 cm^2g^{-1}) as compared to control (573.85 cm^2g^{-1}). Leaf area ratio was high in seedling of control soil (37.30 cm^2g^{-1}) and minimum was recorded in Bhakkar-Notak road soil (25.00 cm^2g^{-1}). Toxic nature of available pollutants in soil usually varied in soil and ultimately effect growth of plants. Reduction trend in different growth variables was not same but changes from soil to soil. Plants do not exhibit similar trend of susceptibility to pollutants. Major variations in response of plants to air born pollutants have been also reported by JACOBSON & HILL (1970). The long period of even low concentration of automobile

pollutants exposure creates destructive effects on seed germination and plant growth with visible injury [JOSHI & SWAMI, 2009].

Table 6. Effects of of different polluted roads site soil on root, shoot, leaf and seeding dry weight of *Albizia lebbeck*

Roads	Root dry weight (g)	Shoot dry weight (g)	Leaf dry weight (g)	Seedling dry weight (g)
A	0.24 ± 0.003b	0.54 ± 0.005b	0.07 ± 0.001ab	0.78 ± 0.008c
B	0.28 ± 0.001ab	0.67 ± 0.004ab	0.08 ± 0.003a	0.95 ± 0.017b
C	0.34 ± 0.002a	0.78 ± 0.002a	0.08 ± 0.001a	1.12 ± 0.013a
D	0.17 ± 0.003bc	0.35 ± 0.003c	0.03 ± 0.002bc	0.52 ± 0.009d
E	0.23 ± 0.004b	0.42 ± 0.002bc	0.04 ± 0.001b	0.65 ± 0.007cd

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error.

Leaf area was significantly ($p<0.05$) high in the seedling raised in control (8.76 cm^2) while other polluted soils showed significant reduction in this parameter. Fresh (1.28 g) and dry weight (1.12 g) of seedling were significantly ($p<0.05$) high in soil of Bhakkar-Jhang road as compared to control (0.91 and 0.63 g respectively). A significant ($p<0.05$) increase was studied in root dry weight of seedlings grown in Bhakkar-Jhang road soil and Bhakkar-Darya Khan road soil recorded as 0.34 and 0.28 g respectively which was greater than control (0.24 g). Bhakkar-Khansar road soil (0.23 g) showed nonsignificant results with control. Maximum shoot dry weight (0.78 g) of seedling was recorded for Bhakkar-Jhang road soil which showed significant ($p<0.05$) result with control (0.54 g). Leaf dry weightof seedlings was high (0.08 g) developed from the soils of Bhakkar-Jhang road and Bhakkar-Darya Khan road when correlated with control (0.07 g). In our findings seedling's fresh weight and dry weight showed significant ($p<0.05$) variations in different polluted roadside soils. POWELL & al. (1996) reported that seedling fresh weight and dry weight got reduced under polluted environment. Both increase and decrease in biomass of seedlings were also recorded by NAWAZ & al. (2006).

Table 7 showed significantly ($p<0.05$) high values of Root shoot ratio in Bhakkar-Khansar road soil (0.55) and Bhakkar-Notak road soil (0.49) as compared to control (0.44). Bhakkar-Jhang road soil (0.44) showed nonsignificant result with control. Seedling developed in control soil showed significant ($p<0.05$) increase in leaf weight ratio (0.09) followed by Bhakkar-Darya Khan road soil (0.08) while prominent reduction was observed in Bhakkar-Khansar road soil (0.06) in relation with control. Highest value of specific leaf area (179.67cm^2g^{-1}) was recorded in the seedling grown in Bhakkar-Notak road soil as compared to control (125.14 cm^2g^{-1}). A considerable amount of Arsenic in air particulates and in diesel exhaust particulates found [TALEBI & ABEDI, 2005]. In comparison, the shoot height and root length of wheat were found more sensitive to arsenic and might be used as indicators for arsenic toxicity [LIU & al. 2005].

Other three roads showed significant low results with control. Leaf area ratio of *Albizia lebbeck* developed in control soil (11.23 cm^2g^{-1}) was greater while other polluted road soil showed significant ($p<0.05$) reduction in this parameter. Among the most important parts of plants, the leaf is the mainly receptive part of plant to be badly pretentious by automobile pollutants. In our research work all parameters related to leaf which includes number of leaves, leaf area, specific leaf area, leaf weight ratio, leaf dry weight and leaf area ratio were reduced significantly ($p<0.05$) in seedlings raised from polluted roadside soil. So, the leaf at all stages

of growth act as best indicator to different automobile contaminants [SHAFIQ & al. 2009]. These pollutants are responsible for stomatal clogging, leaf injury, senescence and reduction in leaf weight [TIWARI & al. 2006]. The reduced leaf area results in reduction of absorbed radiations and subsequently reduction in photosynthesis. Hence, declined in fresh weight and dry weight of leaf is directly interrelated to harmful vehicle pollutants. Our results were supported by the work of SIBAK & GULYAS (1990) noted the decline in leaf size due to automobiles pollutants available in environment.

Table 7. Effects of different polluted roads site soil on root/shoot, leaf weight ratio, specific leaf area and leaf area ratio of *Albizia lebbeck*

Roads	Root/Shoot ratio	Leaf weight ratio	Specific leaf area (cm^2g^{-1})	Leaf area ratio (cm^2g^{-1})
A	0.44 ± 0.005b	0.09 ± 0.01a	125.14 ± 3.64b	11.23 ± 0.20a
B	0.42 ± 0.005bc	0.08 ± 0.02ab	92.88 ± 4.18c	7.82 ± 0.14b
C	0.44 ± 0.007b	0.07 ± 0.02ab	80.25 ± 6.45cd	5.73 ± 0.82c
D	0.49 ± 0.005ab	0.06 ± 0.03b	179.67 ± 3.64a	10.37 ± 0.55ab
E	0.55 ± 0.007a	0.06 ± 0.02b	103.00 ± 2.00bc	6.34 ± 0.45bc

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error.

The seedling growth in terms of root, shoot, seedling height, number of leaves, leaf area, seedling dry weight, root/shoot ratio, leaf weight ratio and specific leaf area ratio performance of *Eucalyptus globulus* Labill. was found different in polluted and non-polluted soils of District Bhakkar (Table 8-10). It might be mainly depending upon nature of pollutants released from automobiles. The significant ($p<0.05$) reduction in shoot length, seedling length, number of leaves and leaf area of *E. globulus* were recorded in the soil of Bhakkar-Notak road as compared to control. Bhakkar-Jhang road soil showed significant increase in root length (5.76 cm) of *E. globulus* as compared to control (4.03 cm). Number of leaveswere significantly ($p<0.05$) high in Bhakkar-Darya Khan road soil (10.23) and Bhakkar-Jhang road soil (9.42) than control (8.80).

Table 8. Growth of *Eucalyptus globulus* in soil of different polluted roads

Roads	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Number of leaves	Leaf area (cm^2)
A	4.03 ± 0.34b	11.59 ± 0.35c	15.62 ± 1.74b	8.80 ± 0.87b	6.40 ± 0.15a
B	4.93 ± 0.66ab	15.57 ± 0.90a	20.50 ± 1.52a	10.23 ± 0.71a	5.23 ± 0.31b
C	5.76 ± 0.62a	13.89 ± 0.56b	19.65 ± 1.32ab	9.42 ± 0.50ab	4.39 ± 0.35c
D	3.11 ± 0.46c	6.97 ± 0.14e	10.08 ± 1.38c	5.80 ± 0.38d	2.18 ± 0.25e
E	2.61 ± 0.57d	9.43 ± 0.70d	12.04 ± 0.40bc	7.20 ± 0.59c	3.87 ± 0.39d

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error.

Root, shoot, leaves dry weight, seedling fresh and dry weight of *E. globulus* significantly ($p<0.05$) decreased in soil of Bhakkar-Notak road (Table 9). A significant ($p<0.05$) increase was observed in fresh (1.05 g) and dry weight (0.86 g) of *E. globulus* seedlings grown in Bhakkar-Darya Khan road soil as compared to control (0.61 and 0.48 g respectively). Root, shoot and leaf dry weight of seedlings grown in different polluted roadside soil showed

significant ($p<0.05$) results with control. Root dry weight (0.24 g), shoot dry weight (0.62 g) and leaf dry weight (0.12 g) was significantly ($p<0.05$) high in Bhakkar-Darya Khan road soil and other polluted road soil showed reduction in these parameters as compared to control (0.16, 0.32 and 0.07 g respectively).

Table 9. Growth of *Eucalyptus globulus* in soil of different polluted roads

Roads	Root dry Weight (g)	Shoot dry weight (g)	Leaf dry weight (g)	Seedling fresh weight (g)	Seedling dry weight (g)
A	0.16 ± 0.006b	0.32 ± 0.005bc	0.07 ± 0.005c	0.61 ± 0.06c	0.48 ± 0.006bc
B	0.24 ± 0.008a	0.62 ± 0.008a	0.12 ± 0.008a	1.05 ± 0.08a	0.86 ± 0.009a
C	0.19 ± 0.006ab	0.40 ± 0.005b	0.08 ± 0.016ab	0.75 ± 0.07b	0.59 ± 0.008b
D	0.09 ± 0.004c	0.16 ± 0.004cd	0.05 ± 0.003cd	0.37 ± 0.03d	0.25 ± 0.009cd
E	0.14 ± 0.004bc	0.21 ± 0.004c	0.06 ± 0.002cd	0.49 ± 0.05cd	0.35 ± 0.006c

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road; **E** = Bhakkar-Khansar road. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error.

The importance to the soil-root-shoot pathway for remediation of contaminated sites with polyaromatic hydrocarbons (PAHs) was reported [SCHWAB & DERMODY, 2021]. Root to shoot ratio, leaf weight ratio, specific leaf area and leaf area ratio of *E. globulus* found influenced by automobile polluted soil treatment. Root/shoot ratio, leaf weight ratio, specific leaf area and leaf area ratio of *E. globulus* in soil of Bhakkar-Darya Khan was recorded (Table 10). Root shoot ratio of *E. globulus* was higher in seedling established from the soils of Bhakkar-Khansar road (0.67) and Bhakkar-Notak road (0.56) when correlated with control (0.50) while Bhakkar-Jhang road soil (0.48) showed nonsignificant ($p<0.05$) results with control. Leaf weight ratio was high in the seedling raised from Bhakkar-Notak road soil (0.21) and Bhakkar-Khansar road soil (0.17) as compared to control (0.15). This parameter presented significant ($p<0.05$) reduction in the soil of other polluted sites. Specific leaf area was significantly ($p<0.05$) highest in control (91.43 cm^2g^{-1}) followed by Bhakkar-Khansar road soil (64.50 cm^2g^{-1}) while other three polluted road side soils showed reduction in this parameter. Leaf area ratio was significantly ($p<0.05$) more in control (13.33 cm^2g^{-1}) followed by Bhakkar-Khansar road soil (11.06 cm^2g^{-1}), Bhakkar-Notak road soil (8.72 cm^2g^{-1}) and Bhakkar-Jhang road soil (7.44 cm^2g^{-1}). Bhakkar-Darya Khan road soil (6.08 cm^2g^{-1}) had lowest value of leaf area ratio.

Table 10. Ratios of different growth variables of *Eucalyptus globulus*in soil of different polluted roads

Roads	Root/Shoot ratio	Leaf weight ratio	Specific leaf area (cm^2g^{-1})	Leaf area ratio (cm^2g^{-1})
A	0.50 ± 0.06bc	0.15 ± 0.003b	91.43 ± 6.76a	13.33 ± 0.69a
B	0.39 ± 0.12c	0.14 ± 0.002bc	43.58 ± 7.03cd	6.08 ± 1.07d
C	0.48 ± 0.17bc	0.14 ± 0.001bc	54.88 ± 2.90c	7.44 ± 0.99cd
D	0.56 ± 0.36b	0.20 ± 0.002a	43.60 ± 5.37cb	8.72 ± 1.06c
E	0.67 ± 0.13a	0.17 ± 0.002ab	64.50 ± 3.90b	11.06 ± 1.40b

Symbol used: **A** = University Sub-Campus road; **B** = Bhakkar-Darya Khan road; **C** = Bhakkar-Jhang road; **D** = Bhakkar-Notak road. **E** = Bhakkar-Khansar road. Numbers followed by the same letter in the same column are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± Standard Error

Changes in soil characteristics influence plant growth and development. The seedling growth performance of *Acacia nilotica* was significantly decreased in polluted soils of Bhakkar-

Khansar road and greater in Bhakkar-Jhang road. Similalrly, a significant decrease in seedling growth performance of *Albizia lebbeck* and *E. globulus* raised in Bhakkar-Notak roadside polluted soils were recorded. The present seedling growth data can be used for the benefits of ecology and improvement in local environment during plantation of vegetation in urbanized, contaminated and degraded soil.

Conclusion

Soil pollution due to release of pollutants from vehicle emissions effects plant growth. It was concluded that due to variation in resistance and sensitivity level to automobile polluted soil some significant changes in growth variables of selected woody plant species was recorded. The results of the present study confirmed that automobile activities polluted the soil of the some Bhakkar area and that make it differently for seedling growth performance of selected three plant species, *Acacia nilotica*, *Albizia lebbeck* and *E. globulus*. *Acacia nilotica* and *Albizia lebbeck* seedlings wereflourished well in the soils of Bhakkar-Jhang road. The seedlings length of *Acacia nilotica* and *Albizia lebbeck* raised in Bhakkar-Khansar and Bhakkar-Notak road soils were highly decreased. The recorded data also showed that seedling length of *E. globulus* were significantly ($p<0.05$) increased in the soil of Bhakkar-Darya Khan road soil and progressively decreased in Bhakkar-Notak road soil. Eco-friendly organizations in the city should be established so that problems of automobile pollution could be brought in the knowledge of citizen. Some advance techniques and enforcement of environmental protection laws should be implemented to reduce the level of automobile pollution.

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EFFECT OF PEG-INDUCED DROUGHT STRESS ON MUNGBEAN PLANTS REVEALED RESISTANT VARIETIES BASED ON LEAF WILTING INDEX AND BIOCHEMICAL MOLECULES

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Abstract: At the early vegetative growth stage, mungbean are mostly affected by drought, and it is also one of the most promising stages that can be used to screen for drought stress tolerance traits in multiple varieties. Therefore, this study utilized polyethylene glycol (PEG-6000) to induce drought stress towards selection of drought tolerance mungbean varieties in their early vegetative growth stage using both hydroponics and soil based systems. In this study, leaf wilting index and responses of biochemical molecules were used as the basic factors to determine the effect of PEG-induced drought stress among the mungbean varieties. Prior to the imposition of drought stress, germination potentials of the varieties were evaluated and all had germination ≥ 60%. Except for Tvr29 and Tvr44, hydroponic system revealed that ≥ 80% of the varieties had ≥ 1 of their leaves significantly ($P \leq 0.05$) wilted. The highest LWI were recorded for Tvr49 and Tvr79. Re-evaluation of Tvr29, Tvr44, Tvr49 and Tvr79 using soil, shows that Tvr29 and TVr44 resisted drought stress. The hydrogen peroxide, superoxide radical and malondialdehyde contents decreased in TVr29 and Tvr44, and increased in Tvr49 and Tvr79 in comparison to the control. Tvr29 and Tvr44 had high proline content than Tvr49 and Tvr79. Based on LWI and biochemical molecules, this study revealed that Tvr29 and Tvr44 should be utilized where water deficit is a challenge to mungbean globally.

Keywords: legumes, plant stress, seed germination, stress tolerance, water stress.

Introduction

Mungbean (*Vigna radiata* (L.) R. Wilczek var. *radiata*) is a short-duration grain legume cultivated across Asia and rapidly spreading to other parts of the world which include Africa and Latin America [KARUPPANAPANDIAN & al. 2006]. The high content of digestible protein, fiber, antioxidants, and phytonutrients [ITOH & al. 2006] has demand mungbean to be in high demand [GHOSH & al. 2015]. However, mungbean's growing environment has become increasingly barren, and drought is the major problem towards mungbean's growth [YIN & al. 2015]. Like any other crop, it responds to a decrease in available soil moisture by reducing its growth and hence productivity [CHAUHAN & al. 2010; SINGH & SINGH, 2011; HANUMANTHARAO & al. 2016]. Yield loss of 31-57% used to occur in mungbean at flowering and 26% at post flowering/podding stages [NADEEM & al. 2019] during drought stress [FATHY & al. 2018]. According to SADASIVAN & al. (1988), drought stress during vegetative phase reduces grain yield through restricted plant size leaf area, root growth, dry matter accumulation, number of pods per plant and low harvest index. Technically, the effects of drought on mungbean begin with osmotic imbalance which gradually develops into metabolic and physiological disorders. These consequently affects photosynthesis

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[SANCHEZ & al. 2012] which is the most important physiological processes that regulate developmental stages in mungbean [ATHAR & ASHRAF, 2005].

Studies have shown variability in morpho-physiological traits for drought tolerance among mungbean varieties during different developmental stages of growth [NARESH & al. 2013; UDDIN & al. 2013]. Apart from the fact that specific changes can occur in plant tissues throughout their life cycle due to drought stress, developing criteria for selection of the best character may be itself a difficult option due to complexity of environment by genotype interactions [MURILLO-AMADOR & al. 2002]. On this note, a better understanding of the responses of mungbean varieties under drought stress condition is required [ABENAVOLI & al. 2016]. Thus, assessment of specific traits and their correlation under drought conditions would be helpful in selecting diverse valuable varieties with defined growth traits [SARKAR & al. 2013; ABRAHA & al. 2015; MISHRA & PANDA, 2017; TIWARI & al. 2018].

Among the various traits, seed germination, seed emergence to seedling stage, leaf damage, chlorosis and genotypic differences within species and leaf wilting have been established for screening drought tolerance traits [RANAWAKE & al. 2012; ALDERFASI & al. 2017; SWATHI & al. 2017] in any crop. Specifically, leaf wilting still remains a fundamental indicator for drought response; and it reduces the complexities associated with drought evaluation in crops. In fact, it was proposed that leaf wilting index (LWI) is the best indicator for crops in their early vegetative growth stage under drought stress [PUNGULANI & al. 2013]. The appearance of leaf wilting can impede photosynthesis as a result of overproduction of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$) and reactive carbonyl species such as malondialdehyde (MDA) [GUO & al. 2012; SUI & al. 2015; HASANUZZAMAN & al. 2017]. Mostly often, under drought stress when ROS level exceeds the defense mechanisms, production and accumulation of H_2O_2 and $O_2^{\cdot-}$ normally enhance MDA which can damage macromolecules, cell structures [FARNESE & al. 2016] and alteration of intrinsic properties of biomolecules and eventually cell death [KURUTAS, 2016]. On the other hand, ROS are tactically exploited as a messenger to activate defense biochemical molecules in plants. Among the biochemical molecules, proline have been established [KAUSHAL & al. 2016] and can be used as criteria to screen mungbean varieties due to the fact proline accumulation is always more than that of amino acids [FAHRAMAND & al. 2014] under drought stress. Based on the above facts on leaf wilting index and plant biochemical molecules, we examined the LWI as the first indicator of drought stress as well as the H_2O_2, $O_2^{\cdot-}$, MDA and proline contents in mungbean varieties in-view to add to reservoir of knowledge on drought stress tolerance mungbean globally.

Materials and methods

Seed germination potential of mungbean varieties

Prior to evaluation of mungbean varieties using Polyethyleneglycol (PEG)-6000, the germination potential of each varieties was determined. Exactly 18 varieties differing in seed morphology and colours were used. The varieties were obtained from Germplasm unit of International Institute of Tropical Agriculture, Ibadan, Nigeria. The seeds were surface sterilized with 0.5% NaOCl for 2 min, followed by 30 sec in 70% ethanol and thoroughly rinsed three times with sterile distilled water. Thereafter, the surface sterilized seeds were allowed to air dry under laminar air flow for 1hr. Exactly 10 seeds were placed at equidistant position in already prepared Petri dish (9 cm – diameter) moistened with two layers of filter papers (Whatman

No.1). This was placed in the dark for 2 days (temperature - 25±2 °C, relative humidity - 65±5%) and later in the light for another 5 long-days photoperiod (16 hr light / 8 hr dark) and maintained under 420 $\mu molm^{-2}$ s^{-1} of photosynthetically active radiation in growth chamber. This experiment was done in three replicates for each varieties. At day 7, seeds were considered to have germinated once the radicle protruded at least 2 mm from testa. The germination percentage was calculated as described by Kader's (2005), (Germination [%] = (number of germinated seeds/total number of seeds) x 100.

Effect of PEG - induced drought stress on mungbean using hydroponics system

Pre-surface sterilized and already germinated seedlings of each varieties were separately and carefully arranged in sizeable netted holes rubber bound to a container (6 cm x 6 cm x 7 cm). Exactly 5 seedlings were positioned to maintain contact with the ¼-strength Hoagland's nutrient solution in the container. Three replicates of each varieties were set up, arranged in randomized complete design and maintained in growth chamber (photoperiod – 16 hr light / 8 hr dark) under the mixture of fluorescent light (about 420 $\mu molm^{-2}$ s^{-1} of photosynthetically active radiation) and incandescent lamps. At 2 days interval, the ¼-strength nutrient solution were regularly changed to prevent algae growth. After emergence of 2 leaves per seedling, the ¼-strength nutrient was supplemented with 20% PEG – 6000 solution. Control seedlings were maintained in ¼-strength nutrients solution (without PEG). The whole experiment (both treatments and control) were allowed to stay for 10 days so that each varieties can maintain interaction with the PEG. A day to termination of the experiment, the LWI was determined using the method described by PUNGULANI & al. (2013). At termination of the experiment, the treated seedlings were again changed into ¼-strength nutrients solution (without PEG) for another 7 days to give room for recovery and the recovery percentage was calculated as the ratio of non-wilted leaves per seedling to that of total number of leaves per seedling.

Effect of PEG - induced drought stress on mungbean using soil

From hydroponics experiment, the two most tolerant (Tvr29 and TVr44) and the two most susceptible (Tvr49 and Tvr79) varieties were selected and re-evaluated in soil using PEG-6000 specifically to re-ascertain their response to drought stress. Pre-sterilized healthy seeds were sown in potted (6 cm – height and 7 cm – diameter) soil. Four seeds were sown into each pot, watered regularly with 20 ml of distilled water and maintained in a growth chamber with temperature of 24±3 °C and relative humidity of 65±5% (photoperiod – 16 hr light / 8 hr dark) under the mixture of fluorescent light (about 420 $\mu molm^{-2}$ s^{-1} of photosynthetically active radiation) and incandescent lamps. At 3-leaf stage, non-uniform seedlings were removed to maintain one seedling per pot. At 4-leaf stage, 20% PEG – 6000 solution was sequentially added on daily basis as 5 ml, 10 ml, 15 ml and 20 ml, and 20 ml was maintained till day 10 while nutrient solution was gradually withdrawn on daily basis as 20 ml, 15 ml, 10 ml, 5 ml and 0 ml was maintained till day10). A day before termination of the experiment, total chlorophyll content [ARNON, 1949], LWI [PUNGULANI & al. 2013] and biochemical molecules were determined from the leaf samples. In addition to the control (without PEG – 6000), the experiment was carried out in three replicates in randomized complete block design. That is, changing the pot positions to reduce environmental factors on the plants. At termination of the experiment, the treatments were re-watered for 7 days and the recovery percentage was calculated as the ratio of non-wilted leaves per plant to that of total number of leaves per plant.

Biochemical assays in mungbean

The proline contents of Tvr29, Tvr44, Tvr49 and Tvr79 were quantified with slight modification in the method described by BATES & al. (1973). Exactly 0.5 g of three replicated leaf samples were homogenized with 5 ml of 3% (w/v) sulfosalicylic acid. Homogenate was obtained by centrifugation using centrifuge (5000 g, 23 °C); 2 ml of the supernatant incubated with 2 ml glacial acetic acid and 2 ml ninhydrin reagent at a ratio of 1:1:1 in a boiling water bath at 100 °C incubated for 30 min. The reaction mixture was allowed to cool down to room temperature, the proline content was assayed through the absorbance of 520 nm. The amount of MDA was determined by the thiobarbituric acid (TBA) reaction in respect to lipid peroxidation according to HEATH & PACKER (1968). Fresh leaf samples (0.2 g) of each mungbean varieties in three replicates were homogenized with 5 ml of 0.25% TBA containing 10% TCA (trichloroacetic acid). The homogenate was subjected to boiling for 30 min at 95 °C and centrifuged at 10,000 g for 10 min. Specific absorbance values at 532 nm was subtracted from values corresponding to non-specific absorption at 600 nm. The MDA content was calculated according to the molar extinction coefficient of MDA (155 mM^{-1} cm^{-1}). H_2O_2 was extracted by homogenizing 200 mg of each varieties tissue separately in three replicates with 5% TCA. The homogenate was centrifuged at 12,500 g for 10 min. To 0.4 ml of 50% TCA, 0.2 ml of (2.5 M) potassium thiocyanate and 0.4 ml of 10 mM of ferrous ammonium sulphate were added with 1.6 ml supernatant to determine H_2O_2 level at the absorbance of 480 nm [SAGISAKA, 1976]. $O_2^{\cdot-}$ was determined based on total O_2 content as described by ELSTNER & HEUPEL (1976). Leaf tissue of 200 mg of each varieties (in three replicates) was homogenized with 5 ml of 65 mM phosphate buffer (pH 7.8). After homogenate was centrifuged at 10000 g for 10 min, 1 ml supernatant, 0.9 ml of phosphate buffer 65 mM (pH 7.8) and 0.1 ml of 10 mM hydroxylamine were added and subjected to 25 °C for 30 min. After incubation, 1 ml of 17 mM sulphinalamide and 1 ml of 7 mM α – naphthyl were added for appropriate reaction at 25 °C. To the reaction mixture, 1 ml of diethyl ether was added and centrifuged at 15,000 g for 5 min and the absorbance was measured at 530 nm. Determination of H_2O_2, $O_2^{\cdot-}$, MDA and proline were independently repeated to ascertain values.

Statistical analysis

Experimental treatments were compared using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). For each experiment, three replicated data sets were subjected to the analysis of variance (ANOVA) technique according to the experimental design to find out the significance of the treatments. ANOVA was also used to determine the effect of treatments and error associated with the experiment. Mean comparison of traits was used and carried out by protected LSD ($P = 0.05$; Students-Newman-Keuls Test) where the error mean square served as the standard error of differences between mean.

Results

Seed germination potentials of mungbean varieties

Germination potential of each mungbean varieties was determined in-vitro under control conditions. All the varieties had ≥ 60% germination. Out of the 18 varieties, 9 (50%) which include Tvr21, Tvr42, Tvr43, Tvr44, Tvr47, Tvr48, Tvr62, Tvr79 and Tvr97 had 100% germination while Tvr29 and Tvr82 had the least germination. Tvr17, Tvr19, Tvr32, Tvr40 and Tvr49 were not significantly ($P \leq 0.05$) different from each other in comparison to Tvr14 and Tvr46 (Figure 1).

Effect of PEG-induced drought stress on mungbean varieties based on leaf wilting index

Under hydroponics system using LWI as indicator of drought stress, observation shows that ≥ 80% of the varieties had ≥ 1 of their leaves wilted. Specifically, Tvr21, Tvr42, Tvr43, Tvr47 and Tvr82 had 50% of their leaves wilted in comparison to their controls. Tvr49 had the highest LWI, followed by Tvr79. On the contrary, Tvr29, followed by Tvr44 exhibited resistance to the PEG-induced drought stress with no evidence of leaf wilting. Although, Tvr46 and Tvr48 demonstrated moderate resistance in comparison to Tvr29 (Figure 2A). After the treatments, we re-engaged the treated plants to ascertain their recovery level. Recovery was maintained for 7 days and percentage recovery was recorded. Tvr29 demonstrated remarkable recovery of 100%, followed by Tvr44 (80%) and Tvr48 (65%) while Tvr49 and Tvr79 were unable to recover. More than 60% of the mungbean varieties had ≤ 40% recovery chances in comparison to Tvr29, Tvr44, Tvr46 and Tvr48 (Figure 2B).

Next, the two most resistant varieties (Tvr29 and Tvr44) and sensitive varieties (Tvr49 and Tvr79) were selected and re-evaluated using soil. Based on observations from soil, the 20% of PEG-induced the highest LWI of 55% and 33% on Tvr49 and Tvr79 respectively. The LWI of Tvr49 was 48% higher than that of Tvr29. Both Tvr29 and Tvr44 had the lowest LWI of 7% and 15% respectively (Figure 2C). The recovery of Tvr29 and Tvr44 in soil was similar to that of hydroponics. Unfortunately, Tvr49 and Tvr79 were unable to recover. Obviously, all the controls recovered irrespective of the resistant or sensitive varieties (Figure 2D). In addition, the effect of the leaf wilting as a result of PEG – induced drought stress also reflected negatively on the chlorophyll of the treated leaves. Specifically, the chlorophyll contents of the controls were relatively insignificant ($P \leq 0.05$) while that of Tvr29 and Tvr44 were outstanding in comparison to the low chlorophyll contents of Tvr49 and Tvr79 (Figure 3).

Phenotypically, apart from the fact that Tvr29 was not showing any sign of leaf wilting, even when compared with the control (without PEG), Tvr29 had a unique leaf rolling morphology pattern. Interestingly, both the PEG treated and the control maintained similar leaf rolling morphology pattern throughout the experiment (Figure 4).

Influence of PEG-induced drought stress on biochemical molecules of mungbean varieties

Biochemical responses of the selected resistant (Tvr29 and Tvr44) and sensitive varieties (Tvr49 and Tvr79) were examined. Under PEG-induced drought stress, the H_2O_2 contents were on the high side in comparison to the control (without PEG). The H_2O_2 content of Tvr49 increased, followed by that of Tvr79 and Tvr29 had the least H_2O_2 content (Figure 5A). Similar observation was recorded for $O_2^{\cdot-}$ content with respect to the examined varieties. All the controls maintain similar trend of significance in comparison to the PEG treated varieties. Tvr49 varieties had a unique $O_2^{\cdot-}$ content in comparison to Tvr79, while Tvr29 and Tv44 had the least (Figure 5B). Both the resistant and sensitive varieties produced proline. However, TVr49 and Tvr79 had low proline content while Tvr29 had the highest, followed by Tvr44 (Figure 5C). In comparison with the control, the MDA content of the Tvr49 was the highest, followed by Tvr79. Tvr29 and Tvr44 had low MDA content (Figure 5D). Generally, H_2O_2, $O_2^{\cdot-}$ and MDA followed similar response pattern where Tvr49 > Tvr79 > Tvr44 > Tvr29. Only proline content followed opposite pattern of Tvr29 > Tvr44 > Tvr79 > Tvr49.

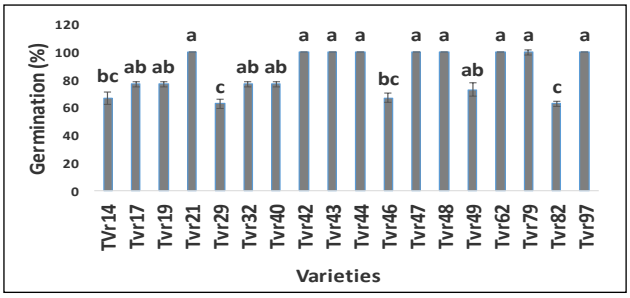

Figure 1. Seed germination potential of each mungbean variety. All the mungbean varieties germinated well (≥ 60%). Means followed by the same letter (s) are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls Test. The results shown are means ± standard error (n=3).

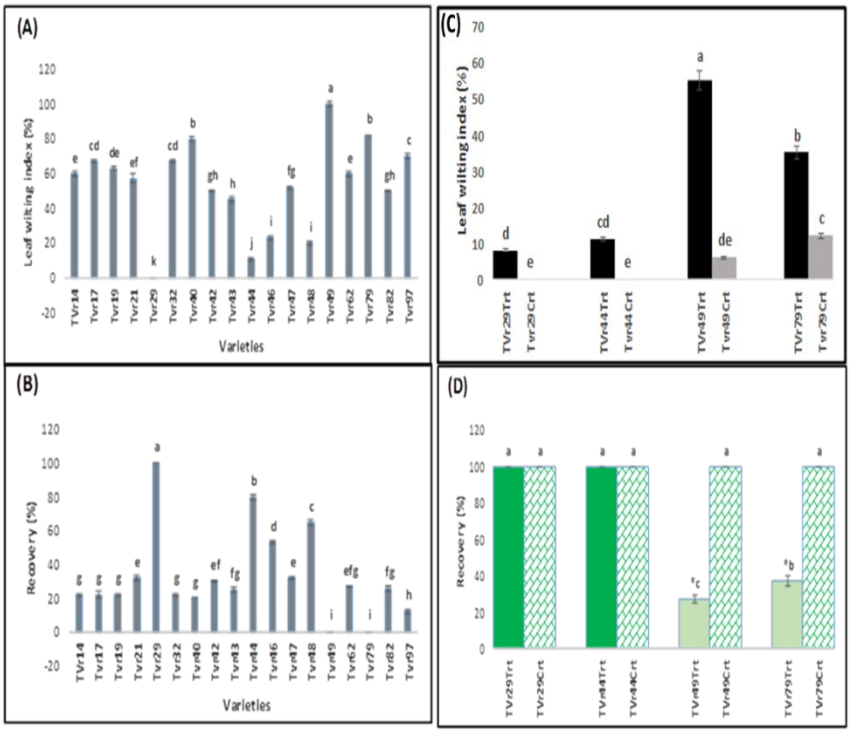

Figure 2. (A) Under hydroponics system, effect of 20% PEG-6000 was determined on leaf wilting and calculated based on leaf wilting index (B) Plant recovery (%) under hydroponics system, (C) Under soil system, effect of 20% PEG-6000 was determined on leaf wilting and calculated based on leaf wilting index, (D) Plant recovery (%) in soil based on effect of PEG-induced drought stress. Tvr29Trt (Tvr29 treated with PEG), Tvr29Crt (Tvr29 without PEG), Tvr44Trt (Tvr44 treated with PEG), Tvr44Crt (Tvr44 without PEG), Tvr49Trt (Tvr49 treated with PEG), Tvr49Crt (Tvr49 without PEG) and Tvr79Trt (Tvr79 treated with PEG), Tvr79Crt (Tvr79 without PEG). (*) indicates significantly ($P \leq 0.05$) sensitive and unable to recover. Means followed by the same letter (s) are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls Test. The results shown are means ± standard error (n=3).

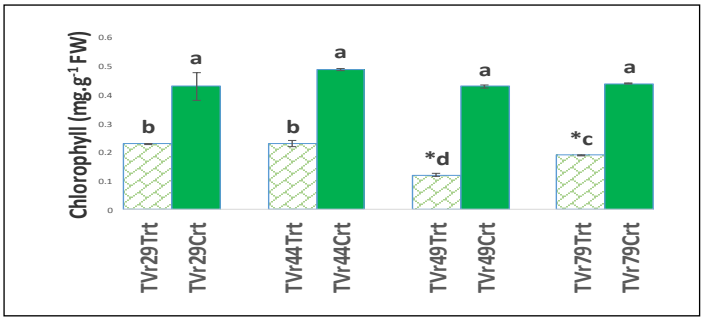

Figure 3. Effect of 20% PEG-6000 on chlorophyll content of selected mungbean varieties (Tvr29, Tvr44, Tvr49 and Tvr79). Tvr29Trt (Tvr29 treated with PEG), Tvr29Crt (Tvr29 without PEG), Tvr44Trt (Tvr44 treated with PEG), Tvr44Crt (Tvr44 without PEG), Tvr49Trt (Tvr49 treated with PEG), Tvr49Crt (Tvr49 without PEG) and Tvr79Trt (Tvr79 treated with PEG), Tvr79Crt (Tvr79 without PEG). (*) indicates significantly ($P \leq 0.05$) sensitive with very low chlorophyll content. Means followed by the same letter (s) are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls Test. The results shown are means ± standard error (n=3).

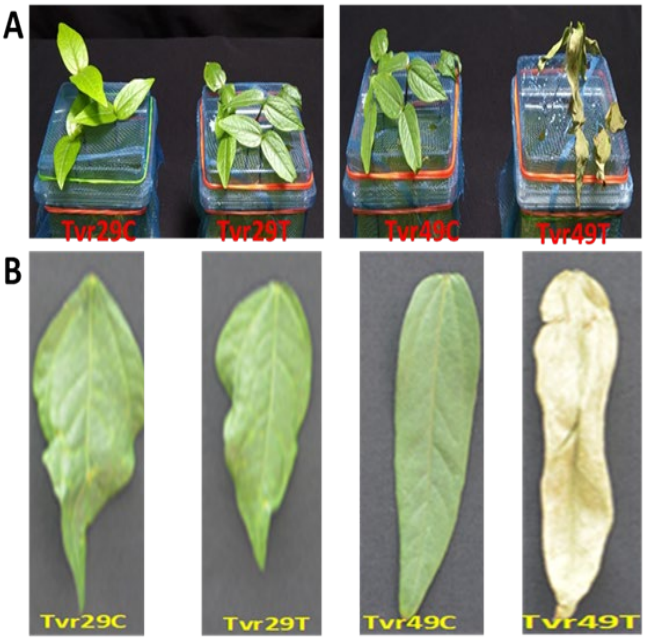

Figure 4. The leaves of TVr29 exhibited resistance to drought stress in comparison to TVr49 that had extreme leaf wilting using (A) hydroponics system and (B) soil system. Tvr29Trt (Tvr29 treated with PEG), Tvr29Crt (Tvr29 without PEG), Tvr44Trt (Tvr44 treated with PEG), Tvr44Crt (Tvr44 without PEG), Tvr49Trt (Tvr49 treated with PEG), Tvr49Crt (Tvr49 without PEG) and Tvr79Trt (Tvr79 treated with PEG), Tvr79Crt (Tvr79 without PEG). Means followed by the same letter (s) are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls Test. The results shown are means ± standard error (n=3).

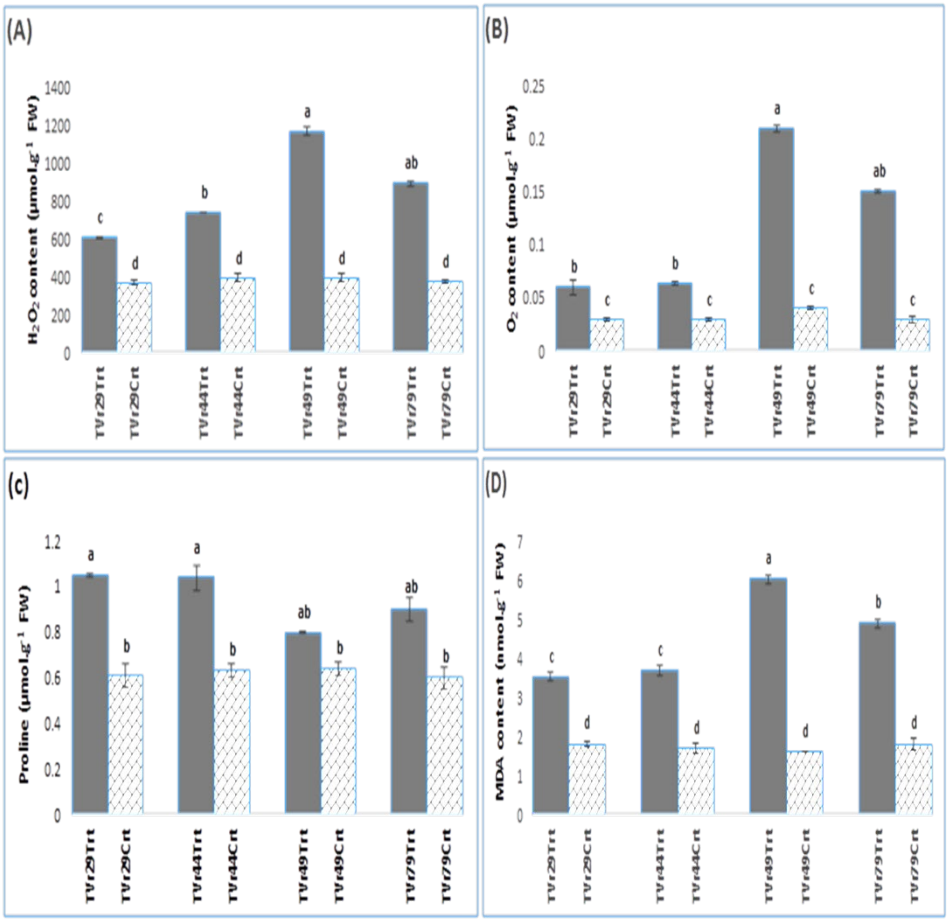

Figure 5. The effect of PEG-induced drought stress on (A) H_2O_2 content, (B) $O_2^{\cdot-}$ content, (C) Proline content, (D) Lipid peroxidation-MDA content, in leaves of mungbean cultivars (Tvr29, Tvr44, Tvr49 and Tvr79) evaluated in experiment carried out in soil system. Tvr29Trt (Tvr29 treated with PEG), Tvr29Crt (Tvr29 without PEG), Tvr44Trt (Tvr44 treated with PEG), Tvr44Crt (Tvr44 without PEG), Tvr49Trt (Tvr49 treated with PEG), Tvr49Crt (Tvr49 without PEG) and Tvr79Trt (Tvr79 treated with PEG), Tvr79Crt (Tvr79 without PEG). Means followed by the same letter (s) are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls Test. The results shown are means ± standard error (n = 3).

Discussion

Seed germination is an important stage in plant development playing crucial roles in seedling emergence and adaptation to environmental factors. Prior to PEG-induced drought stress, seed germination of all the 18 mungbean varieties were evaluated to ascertain the germination potential of each varieties. Their seed germination potential is important, basically, not to misinterpret poor germination for the effect of PEG-induced drought stress. Exactly, 9 out of the 18 varieties had 100% germination, and the remaining 9 had ≥ 60% germination. Although, none of the varieties had poor germination. The potential of a seed to germinate

depends on the ability to utilize the nutritional reserves which was demonstrated among the mungbean varieties [RAO & SINHA, 1993].

Mungbean have the potentials to withstand drought stress [NAHAR & al. 2015; NAIR & al. 2019]. However, extreme drought stress has continued to threaten mungbean production in many parts of the world [ITOH & al. 2006; NAIR & al. 2019]. Both hydroponics and soil based system were conducted using LWI and biochemical molecules such as H_2O_2, $O_2^{\cdot -}$, MDA and proline as response to drought stress on mungbean. For LWI, ≥ 80% of the mungbean varieties had ≥ 1 of their leaves wilted. This implies that, drought can affect any stage of mungbean growth including the early vegetative growth stage [ITOH & al. 2006; BANGAR & al. 2019; NAIR & al. 2019]. Similar observation was reported for early vegetative growth of soybean under PEG-induced drought stress, the soybean varieties at their early vegetative growth had ≥ 1 of their leaves wilted [WANG & al. 2021]. Moderate wilting exhibited by Tvr21, Tvr42, Tvr43, Tvr47 and Tvr82 were relatively important traits in evaluating drought tolerance [PATHAN & al. 2014] but were not considered for mungbean varieties in this study knowing fully well that we are interested in significant demarcation between resistant or sensitive varieties. This is due to the fact that leaf wilting removes complexities and doubt associated with drought tolerance, it is also a fundamental factor that cannot be relegated in phenotyping drought response in crops [PUNGULANI & al. 2013]. In the past, many different leaf wilting indicators have been successfully used [HUANG & al. 1998; OBER & al. 2005; CHARLSON & al. 2009]. However, not without bias as several limitations have been uncovered due to, not only visual and qualitative assessment [PUNGULANI & al. 2013] but also imprecise demarcation between tolerance and sensitive varieties. That was why in our study, Tvr46 and Tvr48 were completely removed and were not either categorized as tolerance or sensitive because they do not have close match with Tvr29 and Tvr44 regarded as tolerant varieties or Tvr49 and Tvr79 identified as sensitive.

In the hydroponics system, wilting was evident and highly pronounced in TVr49 and Tvr79 at day 7 out of 10 days. This findings is not against the observation of PUNGULANI & al. (2013) on cowpea. PUNGULANI & al. (2013) stated that LWI is a better approach for leaf wilting, especially for crops in which wilting is a good indicator for response to drought stress. This was evidence in Tvr49 and Tvr79 at the initial stage of drought stress. The early wilting in Tvr49 and Tvr79 revealed alteration in their physiological characteristics, thus, enhance water loss from the leaf tissues. Previous study have established that early wilting varieties can keep their stomata open immediately they sensed drought [AGBICODO & al. 2009]. This suggest that Tvr49 and Tvr79 could no longer withstand the drought stress imposed by PEG which eventually aggravated extreme wilting for them. The leaves of Tvr49 and Tvr79 lost rigidity, leading to a flaccid state due to increase turgor pressure [TAIZ & ZEIGNER, 2010]. In addition, absence of appropriate drought stress tolerance physiological traits such as stomatal conductance, leaf water potential and osmotic adjustment [SHARMA & KUMAR, 2008] may have trigger the high level of LWI experienced by Tvr49 and Tvr79. Apart from Tvr29 that was highly resistant to PEG-induced drought stress in both hydroponics and soil based systems, insignificant LWI was also recorded for Tvr44. This suggest that both Tvr29 and Tvr44 may have closed their stomata during the initial drought stress. As the PEG-induced stress continue to advance, a unique aperture in stomata opening, high level of water potential and accumulation of osmolytes defense mechanisms could be considered as parts of attributes that maintained Tvr29 and Tvr44.

The recovery followed reverse pattern of the LWI across the varieties. The ability to survive drought stress is an important evolutionary component of plant life. That is, recovery is a crucial component of crop adaptation to drought condition [BLUM, 2011; BLUM &

TUBEROSA, 2018]. The variation observed across the varieties with respect to recovery could be associated with differences in their physiological and biochemical responses [MANE & al. 2008; VASQUEZ-ROBINET & al. 2008; EVERS & al. 2010] of each varieties after drought stress. Notably, the complete wilting in TVr49 and Tvr79 corroborated with the report of TAIZ & ZEIGNER (1998) that most plants are interrupted in their physiological process when the leaf water potentials extremely falls below normal and could results to either low recovery or death of plants. Thus, it is pertinent to note that the morphological and physiological responses of leaves to drought stress are crucial to reduce water loss and promote water use efficiency. When plants sense severe water deficiency, their leaves droop or roll because of loss of cell tugor pressure [POORTER & MARKESTEIJN, 2008]. Leaf rolling as one of the common defense mechanisms in plants against drought stress. Specifically, leaf rolling is a unique mechanism and a drought-adaptation trait induced by turgor pressure [HSIAO & al. 1984] to reduce leaf surface temperature and protects plants from excessive water loss [FANG & XIONG, 2015]. In addition to leave rolling, Tvr29 had smaller and thicker leaves. ESAU (1960) corroborated that among the attributes of drought resistant plants are smaller and thicker leaves expected to have more epidermal trichomes, smaller and denser stomata. This implies that these attributes may have contributed to drought resistant ability of Tvr29. In addition to the fact that plants have developed protective mechanisms to recognize signals allowing them to sense and respond to drought stress, the level of tolerance vary from species to species [HOSSAIN & al. 2015]. In our study, Tvr49 and Tvr79 were extremely sensitive to drought stress, while Tvr29 and Tvr44 exhibited high level of resistant to drought stress [ZHU, 2002]. Most importantly, responses that were expressed on plant growth could be survival or death. However, all plants struggled to adapt by utilizing their adaptive mechanism. Adaptation of plants to drought can be avoidance of tissue water deficits or tolerance of tissue water deficits. Based on our findings, Tvr29 tolerated tissue water deficits due to its small, thicker leaves and maintenance of turgor pressure against drought stress. This observation is in-line with the report of MORGAN (1984) that tolerance of tissue water deficits most commonly involves maintenance of turgor, rigid cell walls or decreased cell size.

The demarcation between Tvr29 and Tvr49 signifies the crucial roles of LWI and recovery to ascertain the status of mungbean under drought stress. This suggest that both LWI and recovery could be useful to mungbean breeders since it can easily demarcate between resistant and sensitive varieties using quantitative index [PUNGULANI & al. 2013]. Also, an early response to drying during which leaf colour changes indicates photosynthetic shutdown. A late response to drying during which leaves fold adaxially and exposed surfaces suggest when respiration ceases and tissues eventually reach an air-dry state [FARRANT & al. 2015] could be responsible for the extreme demarcation in response to drought stress between Tvr29 and Tvr49. Available data have shown that drought stress has the potentials to influence the process of photosynthesis in most plants by adjusting the cell organelles and pigments [MAXWELL & JOHNSON, 2000]. The induced drought stress by PEG significantly reduced the chlorophyll content of Tvr49 and Tvr79. As the drought stress advances, photosynthesis gradually reduced until finally shutdown. As a result of this, the Tvr49 and Tvr79 may have lost their chlorophyll. This agreed with that of FANG & XIONG (2015) of which the decrease in total chlorophyll content can affect growth of mungbean. This could as well be attributed to destruction of chloroplasts and / or instability of the pigment protein complex [KAUR & al. 2016]. Similarly, the chlorophyllase may have increased with direct impact on Tvr49 and Tvr79 varieties [REDDY & VORA, 1986; REDDY & al. 2004]. Tvr29 and Tvr44 retain their chlorophyll content which implies that both of them may have evolved a protective mechanism against the

ROS-induced damage to cellular components which include synthesis of protective pigments. Protection on the integrity of chloroplast membrane is very crucial for the maintenance of the photosynthetic activity of mungbean under drought stress [EFEOĞLU & al. 2009]. Furthermore, the photosynthetic system of Tvr29 and Tvr44 were not destroyed, they were just reversibly inactivated which enables them to recover fast after rehydration [STRASSER & al. 2010]. Therefore, Tvr29 and Tvr44 can be considered to have utilized high energy more efficiently, thus, enhanced water holding ability to avoid damage on exposure to drought stress.

Drought has the potentials to enhance disruption of osmotic balance and over production of ROS like H_2O_2 and $O_2^{\cdot-}$ which used to cause oxidative stress and damage cells [FAIZE & al. 2011; NAHAR & al. 2015]. In wilted leaves, the level of ROS is expected to rise and can lead to permanent metabolic dysfunction and death as observed for Tvr49 [ANJUM & al. 2015]. The redox imbalance due to drought stress increases the rate of metabolism and directly upregulated H_2O_2 and $O_2^{\cdot-}$ production in Tvr49 and Tvr79 [GECHEV & HILLE, 2005; BHATTACHARJEE, 2012]. Further observation suggest that exposure of Tvr49 and Tvr79 to drought stress may have broken the metabolites equilibrium which could have led to oxidative deterioration and eventually cell death [CRUZ DE CARVALHO, 2008]. As a result of this, the membrane phospholipids and fatty acids which are sensitive to overproduction of ROS would have damaged, and resulted to peroxidation of membrane lipids in Tvr49 and Tvr79. H_2O_2, $O_2^{\cdot-}$ and MDA were relatively insignificant in Tvr29 and Tvr44. This indicates that the lower concentrations of H_2O_2, $O_2^{\cdot-}$ [GECHEV & HILLE, 2005; BHATTACHARJEE, 2012; NAHAR & al. 2018] and MDA [MOLLER & al. 2007] were needed for cell signaling and adaptation mechanism [JONES, 2014; OBIDIEGWU & al. 2015]. It was evidence in our study, that Tvr29 and Tvr44 exhibited resistance against drought stress. Apart from other morphological and physiological defense mechanisms, Tvr29 and Tvr44 may have enjoyed bioprotective mechanisms of proline which was significantly ($P \leq 0.05$) expressed in their tissues. Presence of proline in mungbean have been considered as an adaptive strategies to withstand drought stress [BANGAR & al. 2019]. On a more specific note, interaction with scanvenging free radicals and buffering cellular redox [TRIPATHI & GAUR, 2004; BANGAR & al. 2019] are parts of the activities of proline in plants under drought stress and this may have been expressed in TVr29 and Tvr44 due to the high content of proline.

Conclusion

Among the varieties, Tvr29 and Tvr44 exhibited high level of resistance to drought stress in comparison to Tvr49 and Tvr79 that had very low resistance based on LWI and production of H_2O_2, $O_2^{\cdot-}$, MDA and proline. Under PEG-induced drought stress, high level of proline content was remarkably produced by Tvr29, followed by Tvr44. Most importantly, the high proline content and unique leaf rolling morphology were parts of the factors that may have facilitated adaptation of Tvr29 to drought stress in comparison to other varieties. Therefore, Tvr29 and Tvr44 should be evaluated and utilized by breeders and farmers where drought is a challenge on mungbean globally.

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THE EFFECTS OF AUTOMOBILE MOTOR WORKSHOP WASTE WATER ON SEEDLING GROWTH OF KIDNEY BEAN AND MUNG BEAN CROPS UNDER ABIOTIC STRESS

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Abstract: The waste water generation from the automobile motor workshop activities are responsible for environmental degradation in the form of water pollution and showed variable impact on plant growth. This paper gives information, assessment and screening about the effect of motor work shop waste water on seedling growth of two different types of legume bean crops namely, kidney bean (*Phaseolus vulgaris* L.) and mung bean (*Vigna radiata* (L.) R. Wilkczek) in pot culture experiments. The results showed that the increase in treatment of 25%, 50%, 75%, and 100% of motor workshop waste water decreased the root, seedling length, number of leaflets, shoot, leaves dry weight and root/shoot ratio of common bean as compared to control. The waste water treatment of 25% significantly ($p<0.05$) decreased root growth performance and leaves dry weight of common bean as compared to control. The treatment of polluted water produced beneficial and harmful effects on root, shoot length, seedling size, number of leaves, leaf area and biomass production of mung bean. The treatment of waste water at 25% significantly ($p<0.05$) decreased shoot length, leaf area, shoot dry weight, specific leaf area and leaf area ratio as compared to control. Increase in concentrations of polluted water at 50% significantly effects seedling length, specific leaf area and leaf rea ratio of polluted water on mung bean.

The seedlings of *P. vulgaris* and *V. radiata* tested different percentage of tolerance to waste water treatment and found high in control treatment. The results showed that seedlings of *P. vulgaris* showed lowest (32.59%) percentage of tolerance to high concentration (100%) of polluted waste water treatment of workshop. The treatment of polluted water at 25, 50, 75 and 100% decreased the tolerance indices values in seedlings of *V. radiata* by 104.35, 83.37, 67.63 and 63.16 percent as compared to control. The decrease in seedling growth of growth parameter of *P. vulgaris* in this study revealed that it was might be due to abiotic stress produced by waste water.

The chemical analysis of waste water showed gradually increase values of pH (7.61-9.32), electrical conductivity (0.56-2.62 $mScm^{-1}$), chloride (13.60-50.96 mgL^{-1}) and $CaCO_3$ (45.90-65.21 mgL^{-1}) as compared to control.

Keywords: bean crops, growth, motor workshop, phytotoxicity assay, pH, soil.

Introduction

Oil product residues from cars, motorbikes and automotive batteries contain heavy metals, cadmium, nickel, mercury, copper, and other chemical substances produce adverse impact on the plant growth and soil. The discharge of waste water thus pollute underground waters, rivers, and lakes. Insoluble, stable, and slowly degradable oil waste contain toxic chemicals and heavy metals, which enter human body. Sometimes “modern industrial blood” term used for Petroleum [HE & al. 2019]. Automobile service station, repair work shop release a large amount of wastewater which contain many toxic elements such as, fuel, dirt, detergent, solvent, hydrocarbons, heavy metals, total solids, engine oil and greases, chloride, sulfate, organic compounds and responsible for degradation of air, water and soil and effects on life, plant growth and productivity [POTTER & SIMMONS, 1998; ACHUBA, 2006; BONA & al.

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2011; ILEMOBAYO & KOLADE, 2008; KATHI & KHAN, 2011; MAZUMDER & MUKHERJEE, 2011; ASHA & al. 2016]. The effect of waste engine and crude oil pollution on number of plant species, crops, vegetation productivity and soil were observed [ATUANYA, 1987; EKUNDAYO & OBUEKWE, 1997; BENKA-COKER & EKUNDAYO, 1995; AMAKITRI & ONAFEGHARA, 1983; ANOLIEFO & VWIOKO, 1994; ISSOUFI & al. 2006]. Petroleum, heavy hydrocarbons, in contaminated soils showed a serious harm to the soil ecosystem, human health, biota, halophytes, seed germination percentage and biomass yield [KORADE & FULEKAR, 2009; EBADI & al. 2018; LIU & al. 2020; ZEB & al. 2020]. The impact of oil polluted water, petroleum and its products on plant growth required specific concern in ecological studies and can be studied by using bioassay techniques. The studies on plant behavior in petroleum contaminated soils for the identification and selection of oil pollution indicating species was carried out [MARANHO & al. 2009]. A deleterious effect of dielectric fluids in different oil contents 0.5, 1.0, 2.0, 2.5, 5.0, 7.5 and 10% in soil on soybean crop *Glycine max* (L.) Merr. growth was investigated [SANDERSON & al. 2018].

Scientists around the world are working for conserving of water [HUSSIN & al. 2002]. Water pollution is an important problem in many parts of the world. The discharge of waste water from motor workshop into the immediate environment is an important environmental issue and affecting on agronomic crop. Many researchers have drawn their attention on the effects of polluted waste water on growth of bean crops. Therefore, the present study was carried out with the aim to study the effects of waste water on the growth of two commercially an important agronomic legume bean crop, *Phaseolus vulgaris* L. and *Vigna radiata* (L.) R. Wilkczek of Pakistan.

Material and methods

This experimental study was conducted in green house of the Department of Botany, University of Karachi, Pakistan in the month of October having temperature in range of 33/27 °C. Seeds of *Phaseolus vulgaris* L. (white kidneys beans) and *Vigna radiata* (L.) R. Wilkczek (mung bean) were bought from the local seed store of Lahore, city and used for experiments. The waste water from motor transport workshop located at Orangi town was obtained and considered as standard solution of 100%. From the standard solutions further different concentrations as 0, 25, 50, 75 and 100% in distilled water were prepared. Distilled water was used as control for experiment. The seeds were imbibed in distilled water for half an hour to break seed dormancy. The eight seeds were sown in garden loam soil at 1 cm depth in plastic pots of 7.3 cm in diameter and 9.6 cm depth. The pot was kept moist by adding tap water when necessary. Seedlings were allowed to grow for two weeks to reach at a reasonable height. Three best seedlings of equal height were selected and treated with different 0, 25, 50, 75 and 100% concentrations of waste water. This experiment was replicated three times with the design of completely randomized and lasted for four weeks. The height of the plants was measured using a steel scale. The numbers of leaves were counted. The plants were carefully uprooted and the seedlings part rinsed with clean water. The root, stem and leaves were kept in marked paper envelope and finally place in an oven at 80 degree centigrade for 24 hours to obtained dry weight. Oven dried weights for roots, shoot, leaves and total plant weight was recorded. Leaf area were determined as follows:

For leaf area:

Leaf area = Length × Breadth × 2/3

Leaf weight ratio was determined according to the Eq. 1.

Leaf weight ratio = Leaf dry weight/Total plant dry weight. (Eq. 1)

Tolerance indices of seedlings were determined with the help of following formula [IQBAL & REHMATI, 1992].

$$\text{Tolerance indices (T.I.)} = \frac{\text{Mean root length of waste water treated seedlings}}{\text{Mean root length of control seedlings}} \times 100$$

Waste water analysis

The waste water sample was collected from motor transport workshop located at Orangi town motor workshop from Karachi for analysis. Calcium carbonate was determined by acid neutralization as described by ANONYMOUS (1954). pH of waste water was determined by direct pH reading meter (MP-220, Mettler, Toledo). Chlorides were found through titration by Mohr's Method [ALLEN & al. 1974]. Electrical Conductivity (E.C.) were determined by direct AGB 1000 electrical conductivity meter.

Statistical analysis

The means as well as standard errors were calculated. Data collected were subject to one-way analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) using personal computer software packages COSTAT version 3.00 statistically analyzed. Level of significance for these tests was at $P<0.05$.

Results and discussion

Water pollution due to automobile repair workshop is a major global environmental concern both in developing and underdeveloped countries. Water pollution by petroleum products are an important ecological problem likewise air and soil pollution problems. The impact of crude oil into the environment was received worldwide attention [MILLIOLI & al. 2009]. In present study the variable effects of different levels 25%, 50%, 75% and 100% of polluted waste water on the seedling growth performances of legume bean crops, *Phaseolus vulgaris* and *Vigna radiata* and waste water properties were recorded (Table 1). The adverse effect of the various treatments level of polluted water on common and mung bean was measured by assessing through bioassay test. The polluted water affected root, shoot length, seedling size, number of leaves and leaf area as compared to control of both legume crops. These findings are in close conformity with the findings of another researcher's for eggplant, *Capsicum annuum* L. and *Lycopersicon esculentum* Mill. and *Chromolaena odorata* (L.) R. M. King & H. Rob. species [ANOLIEFO & EDEGBAI, 2001; ANOLIEFO & al. 2003; VWIOKO & FASHEMI, 2005; RAHBAR & al. 2012]. The mean root, shoot, leaves, total seedling dry weight, root/shoot ratio, leaf weight ratio, specific leaf area and leaf area ratio of *P*. *vulgaris* and *V*. *radiata* were also highly affected as compared to control treatment. The negative impact of waste water application at 100% on mung bean plant growth as compared to control (without waste water).

The negative effects of engine oil on germination of perennial rye grass and maize growth performance reported [ISIRIMAH & al. 1989; ODJEGBA & SADIQ, 2002; SIDDIQUE

& ADAMS, 2002]. This study demonstrated that treatment of waste water at high concentration in soil has significant effect on the seedling growth performance of *P. vulgaris.* The polluted water treatment at 100% significantly decreased shoot (2.23 cm) and leaves dry weight (0.10 g) of *P. vulgaris* as compared to control. The increase in polluted water level at 75-100% gradually decreased root, seedling height, number of leaflets, root, shoot dry weight of *P. vulgaris* as compared to control (0%) and produced abiotic stress. Abiotic stresses, such as drought, salinity, and heavy metals limit crop productivity and disturb plant growth worldwide [CANTER, 2018; WAQAS & al. 2019].

Table 1. Seedling growth parameter and biomass production of *P. vulgaris* under different concentration (0, 25, 50, 75, 100%) of polluted waste water.

Seedling growth parameters	**Waste water concentration (%)**				
	0	**25**	**50**	**75**	**100**
Root length (cm)	PV7.18±3.20a VR7.20±0.96a	5.33±2.20a 5.88±0.69a	4.17±2.08a 7.20±1.07a	5.40±2.88a 8.17±1.77a	2.34±0.69a 8.12±1.02a
Shoot length (cm)	14.15±2.30b 22.85±1.06c	8.82±2.11ab 18.10±2.44b	7.74±3.87ab 19.30±0.68bc	8.66±4.33ab 15.24±0.96ab	2.23±0.99a 13.27±0.12a
Seedling length (cm)	21.34±5.50a 30.06±0.18b	14.14±4.31a 23.99±2.96a	11.91±5.95a 26.51±1.75ab	14.07±7.09a 23.41±1.12a	4.57±1.45a 21.39±0.89a
Number of leaflets	15.00±3.05a 16.00±1.00a	9.66±1.66a 13.66±1.85a	6.66±3.33a 13.66±1.66a	10.00±5.00a 12.33±2.66a	4.66±1.45a 13.33±1.66a
Leaf area (cm^{-2})	4.88±2.02a 38.53±5.10c	8.14±0.45a 24.60±4.00b	5.30±2.69a 23.56±2.38ab	2.86±1.44a 16.40±1.45ab	3.53±0.84a 12.95±2.95a
Root dry weight (g)	0.04±0.00a 0.02±0.00ab	0.02±0.00ab 0.02±0.00ab	0.02±0.01ab 0.04±0.01b	0.02±0.01ab 0.01±0.00a	0.01±0.00a 0.03±0.00ab
Shoot dry weight (g)	0.04±0.00a 0.04±0.00b	0.03±0.00a 0.03±0.00ab	0.02±0.01a 0.02±0.01a	0.02±0.01a 0.02±0.00ab	0.01±0.00a 0.01±0.00a
Leaves dry weight (g)	0.05±0.00b 0.04±0.00a	0.04±0.00b 0.04±0.00a	0.03±0.01b 0.04±0.00a	0.02±0.01ab 0.03±0.00a	0.01±0.00a 0.15±0.10a
Total plant dry weight (g)	0.13±0.00a 0.10±0.01a	0.09±0.02a 0.10±0.00a	0.04±0.03a 0.23±0.02a	0.07±0.03a 0.07±0.00a	2.24±0.02a 0.20±0.11a
Root / shoot ratio	1.04±0.15a 0.47±0.07a	0.63±0.18a 0.72±0.03a	0.88±0.48a 2.08±0.50c	0.77±0.39a 0.83±0.33a	0.43±0.29a 1.66±0.16bc
Leaf weight ratio	0.37±0.03a 0.44±0.03a	0.45±0.02a 0.40±0.02a	1.67±0.17a 1.70±1.38a	0.23±0.11a 0.46±0.07a	0.60±0.19a 0.61±0.12a
Specific leaf area ($cm^{-2}\ g^{-1}$)	104.85±43.2a 820.00±63.1c	199.38±27.9a 560.65±45.92b	117.96±58.9a 611.99±92.8bc	71.58±36.13a 494.44±30.9b	262.46±150a 194.16±150a
Leaf area ratio ($cm^{-2}\ g^{-1}$)	39.35±17.27a3 65.78±37.5b	90.83±15.78a 229.46±25.6ab	312.16±282a 124.44±63.54a	24.85±12.45a 233.01±40.2ab	176.52±76.36a 97.60±41.16a

Symbol used: PV = *Phaseolus vulgaris* L.; VR = *Vigna radiata* (L.) R. Wilkczek;
Number followed by the same letters in the same row are not significantly different according to Duncan Multiple Range Test at <0.05 level. ± Standard Error

Seedling growth is sensitive to environmental stresses [SULEIMAN & al. 2009]. The variation in the seedling growth parameter in different level of waste water were recorded. An increase in the degradation of oil hydrocarbons occurs in the soils inhabited by the plants [MURATOVA & al. 2003]. SUMATHI & al. (2008) provided an evidence in a study about the adverse effect of the refinery waste on the growth of the *Lens culinaris* Medik. at higher

concentrations. The polluted water treatment significantly decreased root length (5.66 cm), shoot length (8.78 cm), seedling length (14.45 cm), number of leaflets (9.00) and leaf area (12.29 cm^{-2}) as compared to control. Similar trend of decrease in root, shoot dry weight for mung bean was observed. The polluted water treatment at higher concentrations gradually decreased total seedling dry weight (0.09 g), leaf weight ratio (0.31), specific leaf area (447.86 cm^2g^{-1}) and leaf area ratio (142.51 $cm^{-2}g^{-1}$) as compared to control. Polluted contaminated water at higher level (25-50%) affected root growth performance of *V. radiata* due to development of unsuitable growth condition by oil pollution. The contamination with petroleum affects the development of plants due to different physical effects. According to BONA & SANTOS (2003) oil diminishes the soil capacity for retaining water, thus interfering with plant growth.

The effects of waste water on biomass production were also observed. The negative effects of oil contamination on the total biomass of *Avena sativa* L., *Secale cereale* L. and *Hordeum vulgare* L. was observed. MARANHO & al. (2006) investigated the effect of petroleum pollution on the leaf structure of *Podocarpus lambertii* Klotzsch ex Endl. (Podocarpaceae). Our data also showed the influence of oil polluted soil on leaf area of *P. vulgaris* as compared to control. Seedlings biomass of both crops were less productive in oil contaminated water sample and most productive than control soil sample treatment. The results showed that crude oil level (control, 1, 2, 3, 4% W/W), affected fresh and dry weights of the root and the shoot, root volume, stem diameter, number of leaves, leaf area, and stem height affected by ($p < 0.05$) for Eucalyptus [TAHERI & al. 2018]. PETUKHOV & al. (2000) used plant as biotests of soil and water pollution with petroleum and petroleum product. The continuous decrease in seedling growth of growth parameter of *P. vulgaris* in this study revealed that it is due to abiotic stress.

The screening of plant species with their ability to grow on contaminated soil is considered an important step in the planning for phytoremediation program. The seedling growth of *Impatiens balsamina* L. and *Crotalaria retusa* L. was observed in areas adjacent to automobile service stations in Sri Lanka for their tolerance to used lubricating oil (ULO) contaminated soil [GAMAGE & al. 2020]. The seedlings of *P. vulgaris* and *V. radiata* were tested for tolerance in different level to waste water treatment. The presence of heavy metal likewise cadmium might reason for reduction in seedling growth. The use of cadmium (Cd) in agricultural soils transfer to crop plants which can pose a potential health risk to consumers. The concentrations of cadmium in spinach leaves, potato tubers, onion bulbs and wheat grain grown in commercial horticultural operations across New Zealand (NZ) showed that certain soil and environmental factors can be a key influence for determining Cd accumulation in the edible parts of some plant [ZICHENG & al. 2020].

The seedlings of *P. vulgaris* and *V. radiata* showed high percentage of tolerance to waste water treatment in control treatment (Figure 1).

The results showed that seedlings of *P. vulgaris* showed lowest (32.59%) percentage of tolerance to high concentration (100%) of polluted waste water treatment of workshop as compared to control. The seedlings of *V. radiata* showed similar trend of decrease in tolerance with increasing 25, 50 and 100% levels of pollution of motor workshop polluted water as compared to control. The tolerance in seedlings of *P. vulgaris* to polluted water were reduced with the values 58.07% percent when treated with 50% as compared to control. The treatment of polluted water at 25, 50, 75 and 100% decreased the tolerance indices values in seedlings of *V. radiata* by 104.35, 83.37, 67.63 and 63.16 percent as compared to control. The variation in the seedling growth parameter in different level of waste water were recorded. The decrease in seedling growth of growth parameter of *P. vulgaris* in this study revealed that it was might be due to abiotic stress produced by waste water. The analysis of waste water from 0, 25, 50, 75 and 100% showed variation in chemical properties (Table 2).

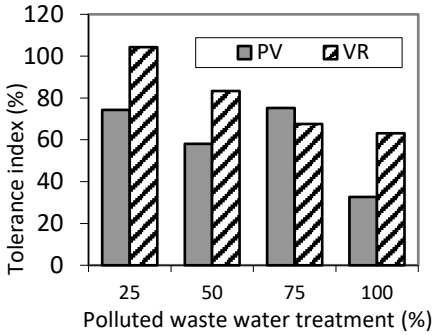

Figure 1. Percentage of tolerance in *Phaseolus vulgaris* L. (PV) and *Vigna radiata* (L.) R. Wilkczek (VR) in different concentration of polluted waste water (25, 50, 75 and 100%) as compared to control.

Table 2. Analysis of motor workshop waste water

	Motor workshop waste water concentration (%)				
Soil parameter	**0**	**25**	**50**	**75**	**100**
pH	7.61a±0.30	8.33b±0.27	8.99ab±0.14	8.57b±0.21	9.32c ±0.10
EC ($mScm^{-1}$)	0.56a±0.23	1.66b±0.00	1.77bc±0.17	2.28bc±0.22	2.62d±0.22
Chloride ($mg\ L^{-1}$)	13.60a±4.19	29.53b±2.90	45.28c±0.98	50.96c±3.40	49.30c±2.50
Calcium carbonate (%)	45.90a±0.50	47.21a±1.74	52.21a±4.76	62.42b±2.36	65.21b±2.65

Number followed by the same letters in the same column are not significantly different according to Duncan Multiple Range Test at <0.05 level. ± Standard Error. Electrical conductivity (EC)

Similar, effect of crude oil pollution from an accidental blowout of an oil well on soil pH, temperature, crude oil content and its flora was studied [DEBOJIT, 2006]. The automobile work shop waste water values gradually increase values of pH (7.61-9.32), electrical conductivity (0.56-2.62 $mScm^{-1}$), chloride (13.60-50.96 mgL^{-1}) and $CaCO_3$ (45.90-65.21 mgL^{-1}) as compared to control (Table 2).

Conclusion

The present study concludes that the significant changes in the seedling growth performances of *P. vulgaris* and *V. radiata* in terms of seedling growth and biomass production was due to treatment of different concentration (25, 50, 75 and 100%) of waste water as compared to control. The reduction was directly proportional to the stress of waste water concentrations. A clear reduction in the shoot, seedling length, number of leaves, leaf area, shoot and leaves productivity of *P. vulgaris* was observed at highest 100% oil polluted water treatment. The significant changes of decrease in shoot length, seedling growth, leaf growth and total seedling dry weight of *V. radiata* grown in the waste water treated soil was also recorded. The waste water bioassay studies can be served as good pollutant indicator of water pollution monitoring.

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INVESTIGATING PROTEIN PARTNERS OF ATMKK1 AS PART OF THE MAPK SIGNALING PATHWAY DURING SALT STRESS

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Abstract: Mitogen-activated protein kinase cascades are one of the many systems that allow plants to survive and defend themselves against pathogens and other environmental stresses. Numerous scientific investigations rendered insights to molecular signaling pathways that take place in an event of a stress such as soil salinity. Despite the known functions and locations of proteins that play a role in these pathways, very little is known about upstream protein partners. In this paper, we elucidate biological functions and molecular locations of *Arabidopsis thaliana* MKK1 protein through data mining predominantly from STRING and BAR databases. Results revealed AtMEKK1 and CRLK1 as upstream protein partners. In addition, AtMKK2 was further analyzed as a redundant protein to AtMKK1.

Keywords: *Arabidopsis*, AtMEKK, AtMKK, CRLK, protein interaction, salt response.

Introduction

The plant kingdom is one of the eukaryotic domains that hugely contribute to oxygen, food, industrial, and pharmaceutical productions. Aside from photosynthetic capabilities, plants are also characterized for their sessile nature. Unlike animals, plants cannot migrate when environmental conditions become intolerant. Instead, they have elaborate systems that enable success and compatibility in a specific habitat. One of these systems is mitogen-activated protein kinase (MAPK) cascades, which are modules that operate through signal transduction in response to environmental and endogenous stimuli. MAPK cascades are crucial in plant growth, development, and more importantly, defense against stresses. Some of the known stresses from which plants are subjected to are pathogens, oxidative stress, extreme temperatures, high salinity, wounding, osmolarity, etc. [ZHANG & KLESSIG, 2001]. The MAPK signaling pathway responds to these threats by either positively or negatively regulating various elements in signal transduction [XING & FOROUD, 2021]. Initiation of the pathway starts with ligand binding to cellular receptors. For an instance, following a pathogen attack, conserved molecules derived from the pathogen called pathogen-associated molecular patterns (PAMPs) interact with plant pattern receptors [PITZSCHKE & al. 2009; XING & FOROUD, 2021]. Then in the downstream signaling process, MAP kinase kinase kinase (MAPKKK or MEKK) activates MAP kinase kinase (MAPKK or MKK) which also then activates MAP kinase (MAPK) [XING & FOROUD, 2021]. The diversity of kinases offers a wide range of activation combinations. And with further complexity brought upon by protein interactions at various levels, plants are able to possess an elaborately versatile system against environmental stresses [HAMEL & al. 2006].

The relay of phosphorylation in MAP kinase cascade is a crucial step in plant signal transduction. Protein phosphorylation is one of the many regulatory processes which takes place in the cellular and molecular level following an exposure to abiotic stress [KUMAR & al. 2020].

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In the case of MAPKs, MAPKKKs transfer phosphate groups to MKKs' activation motifs, serine and/or threonine residues. Then, MKKs phosphorylate threonine and tyrosine (T-X-Y) residues of downstream MAPKs. Once activated, MAPKs further phosphorylate downstream proteins [KUMAR & al. 2020].

Arabidopsis thaliana is a flowering plant that is frequently used in plant biochemistry and molecular genetic investigations. It has been found that *Arabidopsis* has approximately 80 MAPKKKs, 10 MKKs, and 15-20 MAPKs [XING & FOROUD, 2021]. These divergent protein kinases offer a wide variation of combinations in signal transduction. It is important to note, however, that a singular pathway is not unique to one specific stress stimuli. Multiple stresses can trigger activation of the same pathway. For example, the AtMEKK1-AtMKK1/AtMKK2-AtMPK4 pathway can be activated in response to both pathogen attack and salt stress stimuli [CONROY & al. 2013]. This is due to the fact that activation of a specific kinase can actually result in phosphorylation of multiple kinases that are involved in various other pathways. Although identification of signaling pathways has immensely aided in a deeper elucidation of downstream regulatory processes, numerous cascade genes still have unknown functions [CONROY & al. 2013]. In addition, most of the identifications are centred towards downstream proteins but there are limited available insights on upstream protein partners [TEIGE & al. 2004].

In this paper, we elucidate the functional role that AtMKK1 plays in response to salt stress. Through data mining and network analysis, upstream protein partners of AtMKK1 were identified to advance the understanding on cellular and molecular interactors associated in salt response pathways.

Material and methods

Protein-protein interaction partners

Identification of protein partners of AtMKK1 was carried out using the STRING (https://string-db.org). STRING is a database that offers a visual network of protein-protein interactions through physical and functional relations. Currently, the database renders computational network predictions of more than 24 million proteins and 5,000 organisms. Input of the AtMKK1 under the protein name search tab autodetected several matches from 41 organisms, one of which was *Arabidopsis*. Yielded network prediction primarily exhibited query proteins and first shell of interactors associated to AtMKK1. Each predicted partner was analyzed for its function and position relative to AtMKK1 through the information provided on the database itself. Moreover, additional analysis of the interactors was also carried out through redirection from STRING to AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk). This artificial intelligence (AI) system offers protein information about the gene as well as its quaternary structure. Biological functions of the proteins of interest as well as the signaling pathways of which they play a role in are gathered from AlphaFold. Twelve primary protein partners of AtMKK1 were identified and yielded network of predicted interactors was then screen captured from the STRING site.

Co-localization and co-expression

Co-localization and co-expression of upstream protein partners of AtMKK1 were elucidated using The Bio-Analytic Resource (BAR) for Plant Biology website by the University of Toronto (http://bar.utoronto.ca). BAR is a bioinformatic site that offers web-based tools which are mostly centred on genomics and protein-protein interactions. The portal also displays

visualizations of gene expression in 15 plant species. To analyze the three target protein partners, the ePlant browser under Gene Expression and Protein tool was launched. Genes AtMEKK1 (AT4G08500), AtMKK2 (AT4G29810), and CRLK1 (AT5G54590) were entered into the search tab, revealing informative viewers that range from description about the genes to their sequence data. Co-localization of each gene was analyzed in organ, tissue, and cellular levels through a linear score of gene expression. A red colorization of an organ, tissue, and organelle is indicative of high gene expression while bright yellow suggests a linear score of 0, hence no gene expression. On the other hand, co-expression of the genes was identified under the Tissue Specific Root eFP and Abiotic Stress eFP. These particular viewers render visualization of gene expression in specific root tissues as well the plant shoot in response to salt stress.

Data Analysis

Values of gene expression were obtained from the BAR database. In this study, co-expression of the target genes in the shoot and root after salt treatment is shown in scatter plots with smooth lines generated from Microsoft Excel software.

Results

Protein-protein interactions

Twelve proteins were identified as first shell of interactors to AtMKK1 through the STRING database (Figure 1). Two proteins, MEKK1 and CRLK1, were also determined to be upstream protein partners of AtMKK1. Identification of biological function through the AlphaFold database revealed that AtMEKK1 participates in the negative regulation of innate immunity as a defense against pathogens. The protein's location, relative to AtMKK1, is upstream based on the fact that MAPKKKs function upstream and activate downstream MAPKKs [KONG & al. 2012; XING & FOROUD, 2021]. Next, recognition of calcium/calmodulin-regulated receptor-like kinase 1 (CRLK1) as a protein partner of AtMKK1 was carried out independently from the STRING database. As shown in Figure 1, CRLK1 was not established as a primary partner nor was it part of the second shell of interactors. Identification of the function of AtMEKK1 through the AlphaFold site rendered redirection to the Uniprot website which associated a relevant publication about the gene. The article revealed that CRLK1 phosphorylates AtMEKK1 in response to cold stress [FURUYA & al. 2013]. This phosphorylation is indicative of the fact that CRLK1 functions upstream of AtMEKK1 and is therefore also a further upstream regulator of AtMKK1. Lastly, among the first shell of interactors, MKK2 has been previously shown to function redundantly with AtMKK1 in activating the MPK4 pathway [PITZSCHKE & al. 2009]. Through the STRING and AlphaFold databases as well as applicable publications, we identified AtMEKK1, CRLK1, and AtMKK2 as our target genes in investigating upstream protein partners and other equally relevant interactors to AtMKK1 as part of the salt stress signaling pathway.

Co-localization

Co-localization of the three target genes in this study was identified through recognition of their expression in organ, tissue, and cellular levels. Expression of target genes was measured through quantified gene expression levels (GEL) aided by data visualizations. AtMEKK1 is highly expressed in dry seed (216.37), senescent leaf (184.05), cauline leaf (127.5), rosette leaf 4 (131.1), and rosette leaf 2 (124.03) (Figure 2A). On the other hand, high levels of AtMKK2 expression are found in most of the plant's leaves (Figure 2B), with a maximum expression localized in the

proximal half of leaf 7 (400.1). Moreover, the pedicel (322.62) and the sepal (395.77) of flower stage 15 also highly express the AtMKK2 gene. Figure 2C, on the other hand, exhibits high levels of CRLK1 expression (maximum score: 64.52) in all stages of shoot apex, second internode of the stem (49.92), and the entire rosette after transition to flowering (47.7). At this level, AtMEKK1 and AtMKK2 are both highly localized in cauline leaf, senescing leaf, and rosette leaves 4 and 7 while AtMKK2 and CRLK1 co-localize the entire rosette after flowering. Among the three genes, it is apparent that AtMKK2 has the highest expression level while the GELs for CRLK1 range between 0 and 64.5.

At the tissue level, tissues of the root and shoot apex were analyzed. Tissue specific root eFP of the AtMEKK1 gene (Figure 3A) exhibits the highest level of expression in the endodermis (270.28) and phloem pole pericycle (270.23) of the root's elongation zone. Moderately consistent expression of the gene in the endodermis in all zones of the root is also apparent. AtMKK2, however, displays a different expression level pattern (Figure 3B). Highest level of AtMKK2 expression is observed in the columella of the meristematic zone (1637.7). Moderate levels of expression are centred around root tissues of the maturation zone. CRLK1 is highly expressed in the lateral root cap (331.81) of the apical meristem (Figure 3C) and moderate expression levels are seen in the xylem (133.08) of the zone of elongation. Neither of the three target genes share common sites of localization as increased expression of each are distributed in different zones of the root. In the shoot apex (Figure 4), the gene expression level of AtMEKK1 is highest in the leaf abaxial (FIL) (18.71) followed by the rib meristem (WUS) (16.55) while AtMKK2 is highly expressed in the epidermal 1 layer (TML1) (26.98) then in the enlarged peripheral zone (UFO) (24.95). AtMKK2 share a similar level of gene expression with CRLK1 as the gene is also highly expressed in the UFO (5.07). However, its highest level of expression is in the leaf adaxil (AS2) (6.21).

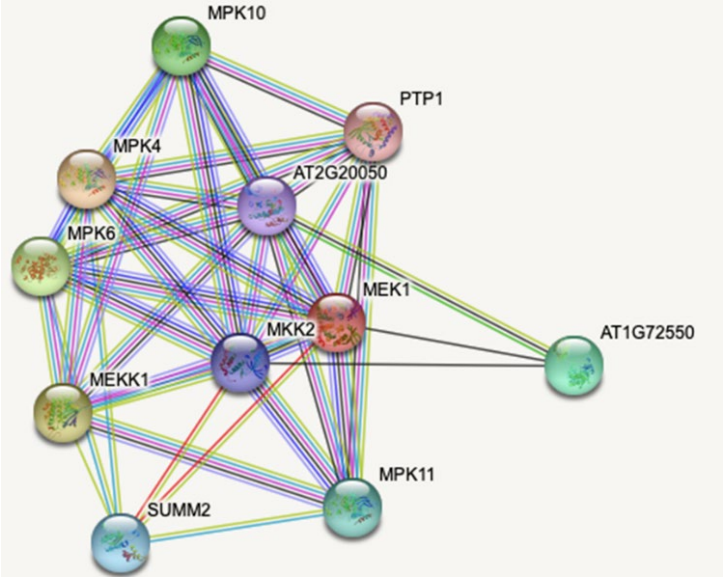

Figure 1. Protein-protein interaction network of the mitogen-activated protein kinase kinase 1 (MKK1) in *Arabidopsis thaliana*. Visual network representation was screen captured from the STRING database.

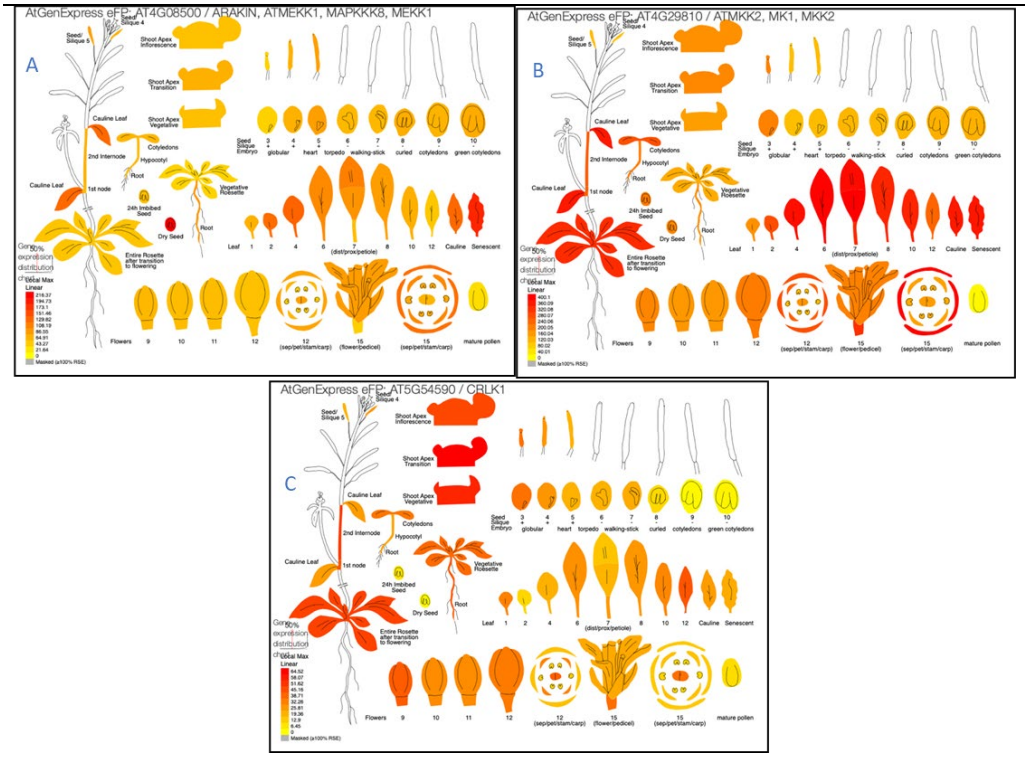

Figure 2. Localization of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* at the organ level. Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Visualization was generated from the Plant eFP viewer of the BAR database.

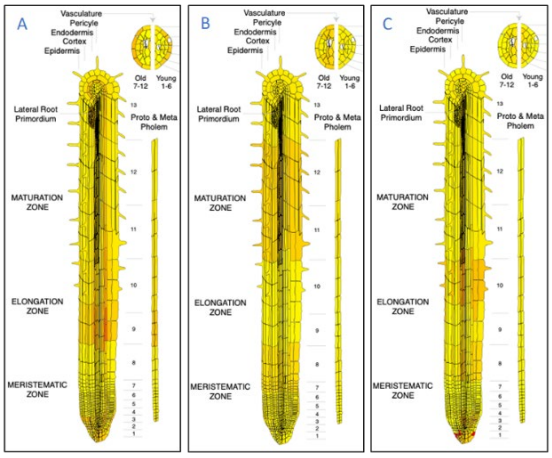

Figure 3. Localization of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* in root tissues. Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Visualization was generated from the Tissue Specific Root eFP viewer of the BAR database.

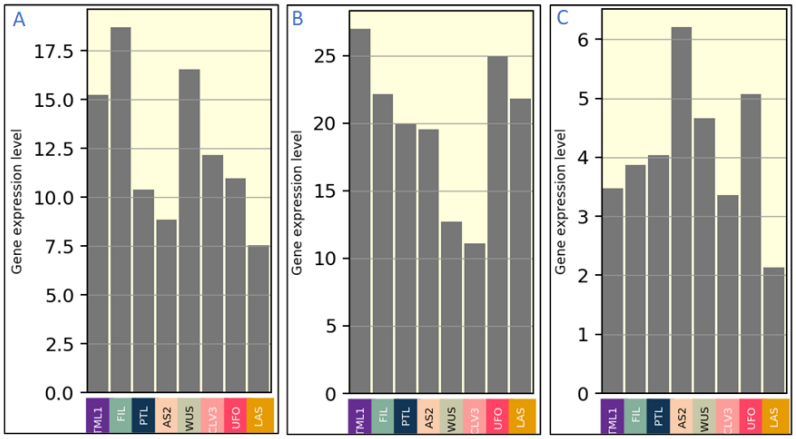

Figure 4. Gene expression levels of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* in various zones of the shoot apex. Charts of expression values were obtained from *Arabidopsis* eFP Browser of the BAR database.

At the cellular level, AtMEKK1 is only highly expressed in the nucleus (32) (Figure 5A). Most of the organelles do not express the gene, with certain exception to the mitochondrion (8) and cytosol (8) for moderate GEL. AtMKK2 exhibits the opposite expression pattern as most of the subcellular locations do express the gene. Highest GEL is in the cytosol (20) while moderate levels are in the vacuole (10), Golgi body (10), mitochondrion (8), and nucleus (6) (Figure 5B). Lastly, CRLK1 is highly expressed in the Golgi body (14), endoplasmic reticulum (14), extracellular membrane (14), and plasma membrane (8) (Figure 5C). Moderate levels of GEL are in the nucleus (6) and cytosol (4). At this level, both the AtMKK2 and CRLK1 are highly expressed in the Golgi body and moderate expression of these genes are also seen in the nucleus. AtMEKK1 and CRLK1 do not share any cellular co-localization, however.

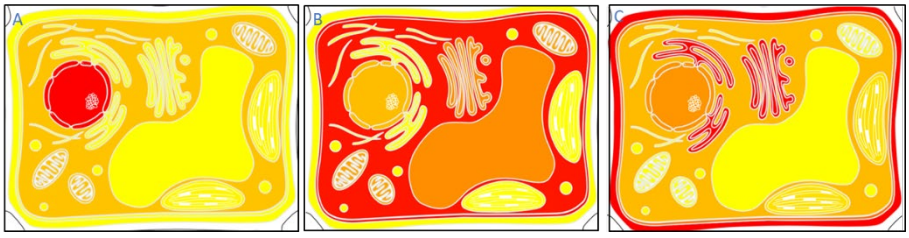

Figure 5. Gene expression levels of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* at the subcellular level. Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Images were generated from Cell eFP viewer of the BAR database.

Co-expression

Co-expression of the three target genes was identified through BAR's Abiotic stress eFP and *Arabidopsis* eFP browser. In the experiment concerning each gene, *Arabidopsis* was grown under environmental conditions with 150 mM salt (NaCl). Gene expressions were measured at seven varying times with hour 0 and hour 24 post-exposure. Expressions of the

genes in the shoot and root are visually represented (Figure 6). Starting with AtMEKK1, at hour 0, GEL in the shoot is 54.59 while GEL in the root is 54.52. Gene expression levels in both the root and the shoot immediately increased 30 minutes after salt exposure, however, past 0.5 hour, patterns of expression levels varied between the two. Expression of AtMEKK1 was at its highest 1-hour post salt exposure then decreased until hour 6 where expression levels went back up to baseline (Figure 7A). After 24 hours, GEL in the shoot was 59.64. AtMEKK1 expression levels in the root decreased after hour 1 but a sudden increase is seen at hour 6 where a maximum GEL of 249.75 was reached. Past the hour of 6, expression levels decreased and a GEL of 144.68 was recorded after 24 hours post-treatment. Expression levels of the AtMKK2 (Figure 7B) follow a similar pattern as AtMEKK1. At hour 0, the shoot has a GEL of 151.77 which increased after an hour of salt treatment. Following a decrease at hour 3, GEL increased until a maximum level of 244.43 was achieved at hour 12. In the root, AtMKK2 expression level at hour 0 was 205.61. An increasing trend can be seen after salt exposure up to a maximum level of 432.75 at hour 6. Baseline levels were achieved as GEL decreased and after 24 hours, recorded data was at 264.49. Expression levels of CRLK1 in *Arabidopsis* (Figure 7C) in response to salt stress is varied among the other two target genes. In the shoot, the GEL at hour 0 started off at 53.81, which was the highest recorded level within the 24-hour period. From here, GEL had decreased until it hit its lowest value at hour 6 (37.52) then proceeded to increase at hour 12 (49.7). A GEL of 29.12 was recorded 24 hours post-treatment. In the root, the value at hour 0 was 31.93 which immediately increased 30 minutes after salt exposure. This increase was the highest expression value for the CRLK1 gene. A decrease can be seen at hour 3 but values increased to baseline levels until the 24-hour period was reached.

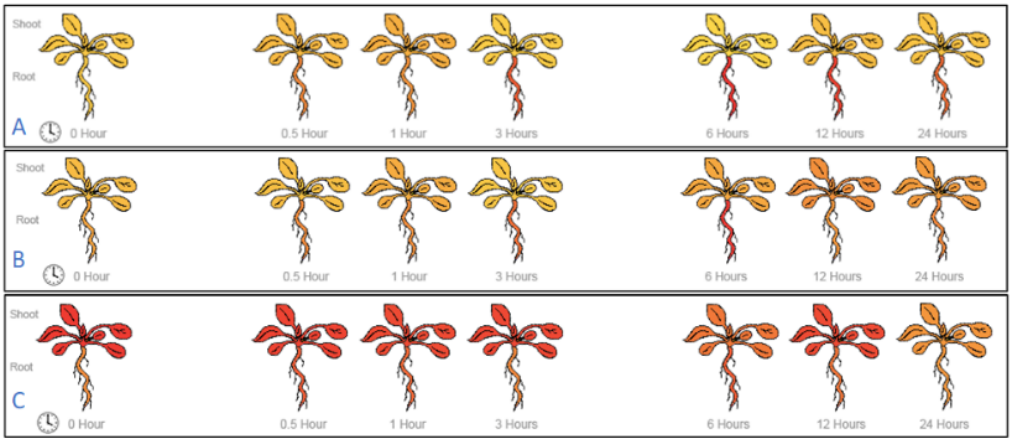

Figure 6. Visualization of gene expression levels of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* after treatment of 150 mM salt (NaCl). Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Images were obtained from the *Arabidopsis* eFP browser of the BAR database.

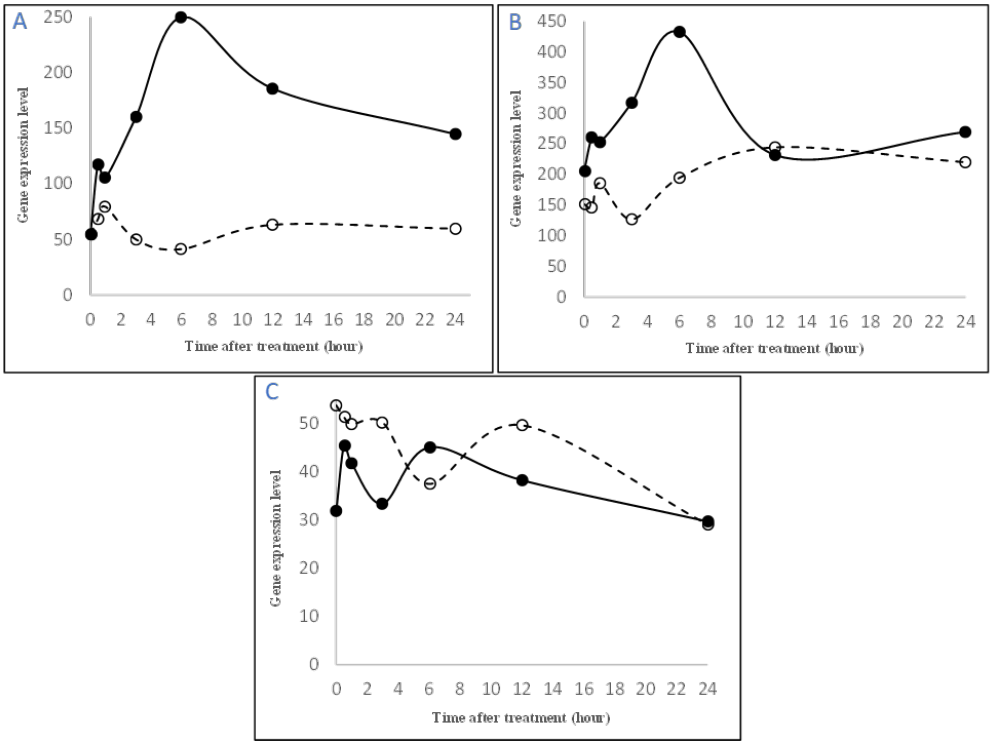

Figure 7. Gene expression pattern of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* after treatment of 150 mM salt (NaCl).

Discussion

Mitogen-activated protein kinase cascades immensely contribute to a plant's defense system against stresses, predominantly the environmental ones. For instance, salt stress has been known to affect productivity and growth in plants as it induces a subgroup of strains such as ionic, osmotic, and oxidative stresses [YANG & YUO, 2018]. Recent investigations unfolded an increase in soil salinity as a result of aggravating industrial pollution, excessive fertilizer use, and faulty irrigation practices [YANG & YUO, 2018]. Cellular and molecular mechanisms of plants' defense systems against biotic and abiotic stresses have been one of the major investigative subjects. In this study, we elucidated upstream protein partners of AtMKK1 to advance the understanding on the limited insight concerning salt signaling pathway in *Arabidopsis*. Data mining through bioinformatic tools like STRING and BAR aided in the investigation of interactors. Starting with STRING, network visualization of protein-protein interactions revealed 11 proteins. Among these, AtMEKK1 was identified as an upstream regulator and AtMKK2 established relevance as a redundant protein to AtMKK1. Further data mining through AlphaFold and Uniprot also unfolded another protein partner, CRLK1. Localization of these target genes through BAR displayed expression in many organ, tissue, and subcellular locations. Despite varying intensity in gene expressions across different locations, the observation that these genes are in fact vastly distributed in many plant tissues secures the

expectation that they are able to transduce signals more productively. In addition, knowing their presence in extensive tissue locations also suggests that these genes can be expressed into proteins for the purpose of inducing various biological processes. On the other hand, co-expression of the target genes revealed that their expression levels after salt treatment predominantly increased. This observation again supports the notion that they play biological roles in the defense signaling system against salinity.

Downstream MAPK cascades are heavily dependent on phosphorylation of proteins to amplify specific stimuli and elicit a physiological and biochemical response. Understanding protein functions and the mechanism behind their activation are imperative in this built-in system. A previous study has established that AtMEKK1 expression enabled prolonged survival of yeast under intense salinity as a result of increased glycerol synthesis [COVIC & al. 1999]. The researchers also observed that among cold, water, and salt stresses, highest levels of AtMEKK1 expression was induced by salt stress [COVIC & al. 1999]. More importantly, AtMEKK1 phosphorylated downstream AtMKK2 [FURUYA & al. 2013]. Molecular evidences suggested that AtMKK2 shares similar and specific functions with AtMKK1 [QIU & al. 2008]. Among other molecular roles, both proteins interact with downstream MPK4 which is a protein associated with the jasmonate (JA) signaling pathway [TEIGE & al. 2004]. The JA pathway has been found to inhibit cell division and elongation in the root tissues in order to steer clear of high salt concentration in the soil [VALENZUELA & al. 2016]. Finally, CRLK1 has been demonstrated to interact with MEKK1 in the regulation of MAPK cascades during cold stress [YANG & al. 2010]. This establishes relevance in this study as CRLK1 could potentially be implicated in the salt stress signaling pathway as well, given the overlap in the activation cascade following MEKK1 phosphorylation between cold stress and salinity.

Dependence on plants as sustenance is one of the reasons behind growing scientific investigations that aim to better plant production and resistance against stresses. Today, research on creating genetically engineered plants is increasing. Advancing our understanding on molecular mechanisms behind the elaborated plant systems that enable defense and resistance can potentially lead us closer to filling the gap and using findings to genetically modify plant species, especially those that are more prone to stresses.

Acknowledgements

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CELL DEATH AND ANTI-CELL DEATH IN TWO WHEAT CULTIVARS AND THE IMPLICATION OF THEIR INVOLVEMENT IN DISEASE RESPONSE

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Abstract: Cell death occurs under various developmental and stress conditions. Its involvement in plant response to pathogen attacks has been well studied in model plant *Arabidopsis thaliana.* In our present work, Fumonisin B1, a toxin from *Fusarium verticillioides*, a major fungal pathogen of cereals, was used as a biotic stressor to trigger responses in two wheat cultivars. Fumonisin B1 induced cell death in both Fusarium head blight (FHB) resistant and FHB susceptible cultivars (Frontana and Roblin, respectively). The treatment also triggered DNA smearing in both. However, the expression of two DNA repairing genes was enhanced in Frontana but not in Roblin. Our results have suggested potential regulatory differences in the response to FB1 toxin in FHB resistant and FHB susceptible cultivars.

Keywords: cell death, DNA repair, Fumonisin B1, *Fusarium* head blight, wheat.

Introduction

Programmed cell death (PCD) is recognized as an essential physiological and genetic process during plant development and in response to biotic and abiotic stresses [BEERS & MCDOWELL, 2001; DANEVA & al. 2016]. Localized cell death occurs in both susceptible and immune plants during pathogen attack. In immune plants, a host resistance (R) protein recognizes a pathogen effector leading to hypersensitive response (HR), which is a form of localized PCD [BURKE & al. 2020; POZO & al. 2004]. Many host responses precede the HR, including proteolysis, changes in ion fluxes, production of reactive oxygen species (ROS), and activation of mitogen-activated protein kinase (MAPK) cascades. In susceptible plants, much less is known about the molecular events leading to cell death. Studies suggest that host-controlled PCD plays a role in cell death occurred in different plant tissues [COLL & al. 2011; GREENBERG & YAO, 2004]. For biotrophic pathogens, early activation of host PCD would likely limit pathogen spread, whereas necrotrophic pathogens benefit from host cell death and kill the host by injecting toxins or activating host PCD [COLL & al. 2011; POZO & al. 2004].

Specific DNA fragmentation into oligonucleosomal units or DNA laddering occurs during PCD in both animal and plant cells [DANON & al. 2000]. Necrosis and a DNA smear on agarose gels are normally caused by concurrent nuclease and protease activities [DANON & al. 2000; WYLLIE & al. 1980]. DNA laddering phenomenon has been studied for a long time in different plant developmental processes and under environmental stresses such as cold [KOUKALOVÀ & al. 1997], UV radiation [DANON & GALLOIS, 1998], and heat [FAN & XING, 2004; SWIDZINSKI & al. 2002]. Harsh environmental stresses often cause necrosis that is an accidental cell death, accompanied with DNA smear, rupture of nuclear, organelle and

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plasma membranes [DANON & al. 2000]. Therefore, DNA ladders are currently used to distinguish apoptosis from necrosis at the molecular level [DANON & al. 2000].

Fusarium verticillioides produces Fumonisin B1 (FB1) [ASAI & al. 2000] and it was used as a biotic stressor in our present study. In *Arabidopsis*, FB1 treatment initiated nuclear DNA fragmentation preceding the loss of membrane integrity, which resembled apoptosis typically associated with PCD in animal cells [ASAI & al. 2000; PLETT & al. 2009]. FB1 induced cell death is dependent on active transcription and translation, as well as reversible protein phosphorylation [ASAI & al. 2000]. In *Arabidopsis* protoplasts FB1-elicited PCD was shown to required SA, jasmonic acid (JA) and ethylene (ET)-dependent signaling pathways as well as one or more unknown factors activated by FB1 [ASAI & al. 2000]. There was also a correlation between MAPK activity and cell death during plant-pathogen interactions [POZO & al. 2004; YANG & al. 2001; ZHANG & LIU, 2001]. Our previous study has indicated that *Arabidopsis* five ethylene receptors have different roles in FB1-induced cell death [PLETT & al. 2009]. Here, we have shown that FB1 induced cell death in both FHB resistant and FHB susceptible wheat cultivars (Frontana and Roblin, respectively). Our results have also suggested potential regulatory differences in the response to FB1 toxin including the expression of anti-cell death genes.

Materials and methods

Plant materials and growth conditions

Wheat (*Triticum aestivum*) seeds were sterilized in a solution of 70% ethanol for 2 min, then transferred to a bleach solution of 25 mL of bleach, 25 mL of distilled water and 10 µL of Triton X-100. The seeds were then rinsed 10 times in distilled water. After drying 5-6 seeds were potted in autoclaved Pro-mix BX soil fertilized with 7-9 granular of slow release NPK fertilizer (14:14:14). The seeds were then placed in a growth chamber (Enconnair Technologies Inc., Winnipeg, MB, Canada) set for 16 hrs at 22°C in the light and 8 hrs at 18°C in the dark. Plants were watered every second day.

FB1 treatment

Frontana and Roblin leaves were collected and treated with FB1 for different periods of time. Three to four leaves harvested from the 3-week-old plants were cut to ~2 cm segments and incubated in 5 µM FB1 at same growth conditions after infiltration. Leaf segments were stretched upward on the filter paper in Petri dish so that they were just covered by the solution. Control leaf segments were treated in the same way with an equal volume of distilled water. Samples after 24 hrs, 48 hrs and 72 hrs incubation were collected. The leaf segments without any treatment were taken as the sample at 0 hr for both FB1 and water treatment. Leaf segments were collected in Falcon tubes, and directly used for staining. For DNA or RNA extraction, leaf materials were collected in Falcon tubes and snap frozen in liquid nitrogen. The materials were then stored at -80 °C till use.

Trypan blue staining

Cell death was detected using an Axioplan 2 microscope (Carl Zeiss, Germany). Methods described by TANG & al. (1999) and STONE & al. (2000) were used with slight modifications. Leaf tissues were immersed in 10 mL of ethanol-lactophenol (2 volumes of ethanol and 1 volume of phenol-glycerol-lactic acid-water 1:1:1:1) that contained 0.05% trypan blue. The leaves were placed in 15 mL Falcon tubes and covered with ethanol-lactophenol-

trypan blue. The samples were incubated at 95 °C for 4 min and then kept at room temperature for 20 min. The staining solution was removed and 1.5 mL chloral hydrate destaining solution (2.5 g/mL of nano pure water) was added to each tube. The leaves were cleared for 2 days by replacing the destaining solution twice. After destaining, leaves were suspended in 50% glycerol and examined under microscope with white light.

Aniline blue staining

Wheat leaves were immersed and vacuum-infiltrated in 10 mL of ethanol-lactophenol (2:1 v:v) and then incubated at 60 °C for 30 min. Leaves were rinsed in 50% ethanol and stained overnight with aniline blue (0.01% aniline blue powder in 150 mM K_2HPO_4, pH 9.5). Leaves were equilibrated in 50% glycerol and aniline blue staining was visualized using a UV epifluorescence (Axioplan 2 microscope, Carl Zeiss, Germany) with a DAPI filter.

Isolation of nuclear DNA and DNA laddering analysis

Total DNA was isolated using a modified method of FAN & XING (2004). Briefly, wheat leaves were ground to a fine powder in liquid nitrogen and added to the extraction buffer (200 mM Tris, pH 7.5, 25 mM EDTA, and 0.5% sodium dodecylsulphate). The supernatant was extracted with phenol and chloroform before precipitation with isopropanol. The DNA solution was incubated at 37 °C for 1 hr in the presence of RNase and 10 µg DNA was separated by electrophoresis in a 2% agarose gel. The gel was then stained with ethidium bromide for visualization of DNA.

Expression analysis of DNA repair genes

RNA was extracted from wheat leaves. GAPDH gene (GenBank accession number EU022331.1) was used as an internal standard [LLOYD & al. 2007]. For RT-PCR, the primers were 5'-GTGAGGCTGGTGCTGATTACG-3' (forward) and 5'-TGGTGCAGCTAG CATTTGAGAC-3' (reverse). The following conditions were used in RT-PCR for wheat GAPDH gene: 94 °C for 1 min; 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 seconds for 28 cycles; and then 10 min at 72 ° C. The size of the amplified fragment was 198 bp.

The primers for RAD50 (GenBank accession number EU159424.1) were 5'-CAGGGACACATTGACTGGTG-3' (forward) and 5'-TTTCCTCGGCAAAATGTACC-3' (reverse). The following conditions were used for RT-PCR: 94 °C for 1 min; 94 °C for 1 min, 67 °C for 1 min, 72 °C for 30 seconds for 28 cycles, and then 72 °C for 10 min. The size of the amplified fragment was 176 bp. The primers for RAD51 (GenBank accession number EU915557.1) were 5'-CAGAAGGCACATTCAGACCA-3' (forward) and 5'-GCAAACCTTG TCTCCACCAT-3' (reverse). The RT-PCR setting was 94 °C for 1 min; 94 °C for 1 min, 71 °C for 1 min, 72 °C for 30 second for 28 cycles, and then 72 °C for 10 min. The size of the amplified fragment was 166 bp.

Results

FB1-induced cell death in Frontana and Roblin leaves

Cell death levels were determined after leaves were detached from three-week-old Frontana and Roblin and treated with water or 5 µM FB1 in long day conditions for up to 72 hrs. Trypan blue is commonly used to selectively stain dead tissues or cells blue, and under white light the dead cells appeared to be much darker compared to living cells. These blue dead cells scattered on leaves as clusters without defined margins and the cell death did not seem to

occur in the whole leaf. Microscopic images were taken by focusing on one of several dead cell groups. Water treatment was used as a control for all plants and no dead cells were detected (Figure 1A). Upon FB1 treatment, cell death was first observed at 24 hrs in both Frontana and Roblin, and more dead cells were detected when leaves were incubated longer (Figure 1A).

The accumulation of callose deposition around the site of infection is usually part of the complex cell wall-strengthening process that halts pathogen invasion [WANG & al. 2021]. To determine whether the cell death detected by trypan blue assay is associated with callose deposition, we determined the presence of callose with aniline blue in Frontana and Roblin leaves 0 hr, 24 hrs, 48 hrs, and 72 hrs after FB1 treatment. In water control, cell death was induced at 72 hrs, and the area of dead cells is larger on Roblin leaves than on Frontana leaves (Figure 1B). The earliest cell death induced by FB1 was detected at 24 hrs in both Frontana and Roblin, and more cell death appeared at 72 hrs (Figure 1B). Overall, upon FB1 treatment, cell death occurred earlier in Frontana leaves than in Roblin leaves as detected by trypan blue and aniline blue, but there was more cell death in Roblin leaves than in Frontana leaves at 72 hrs.

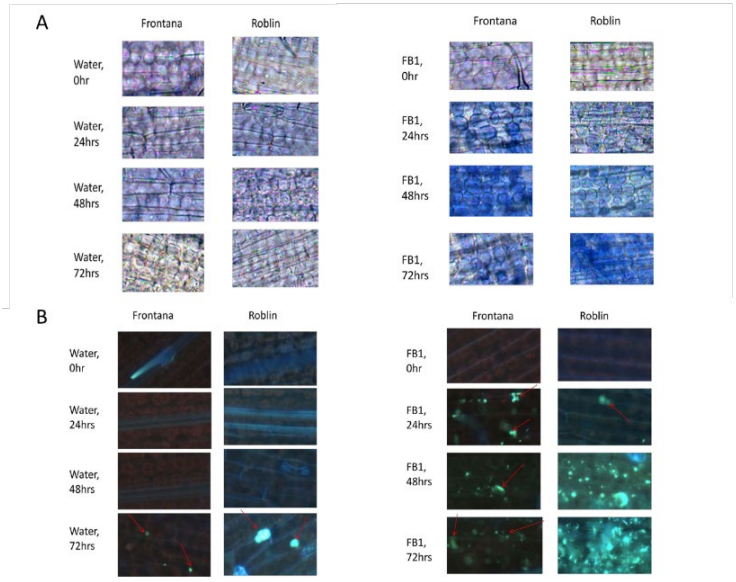

Figure 1. Microscopic images of Frontana and Roblin leaves. Leaves detached from three-week-old plants were treated with water or 5 μM FB1. Half of each type of leaves was then stained with trypan blue. Microscopic images were taken with white light by Axioplan 2 microscope. The other half portion of leaves was stained with aniline blue. Microscopic images were taken using a microscope equipped with a UV light source and DAPI filter (scan range: 320-520 nm). Arrows indicate the sites of fluorescent signals from aniline blue strain. A. Trypan blue detection of cell death. B. Aniline blue detection of cell death. Three experiments were carried out with similar results.

FB1 induced DNA smearing in Frontana and Roblin leaves

DNA ladder phenomenon is one of the most common characteristics of PCD in animals [NING & al. 2002]. We monitored the integrity of DNA by electrophoresis. When Frontana and Roblin leaves were treated with FB1 for different periods of time, a significant DNA smearing was observed (Figure 2).

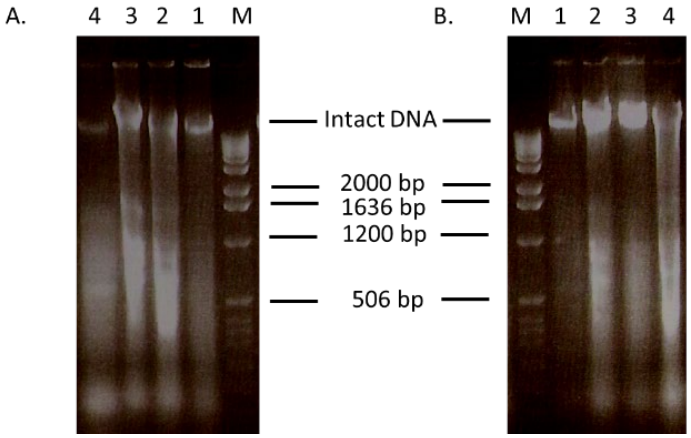

Figure 2. Wheat leaf DNA smearing after FB1 treatments. Ten µg DNA per lane was loaded onto a 2% agarose gel followed by electrophoresis. **A**. Frontana. Lanes 1-4, Frontana leaves after FB1 treatment for 0, 24, 48 and 72 hrs, respectively. **B**. Roblin. Lane 1-4, Roblin leaves after FB1 treatment for 0, 24, 48 and 72 hrs, respectively. M, DNA markers. Three experiments were carried out with similar results.

Expression of DNA repair genes in Frontana and Roblin leaves treated with FB1

DNA repair genes including Radiation sensitive 50 (RAD50) and Radiation sensitive 51 (RAD51) have been identified in yeast, animals and plants, and they are involved in various processes such as DNA damage repair, DNA replication, meiosis, and telomere maintenance [CZORNAK & al. 2008; LAMARCH & al. 2010; LLOYD & al. 2007]. In order to examine whether RAD50 and RAD51 are involved in DNA damage repair after a pathogen attack, DNA integrity of three-week old Frontana and Roblin leaves treated with water or 5 µM FB1 for 0, 24, and 48 hrs was examined. There was no significant change in the expression levels of either RAD50 or RAD51 in Roblin. However, there was an increase in the transcript levels of RAD50 and RAD51 in Frontana at the 24 hrs time interval after the FB1 treatment (Figure 3).

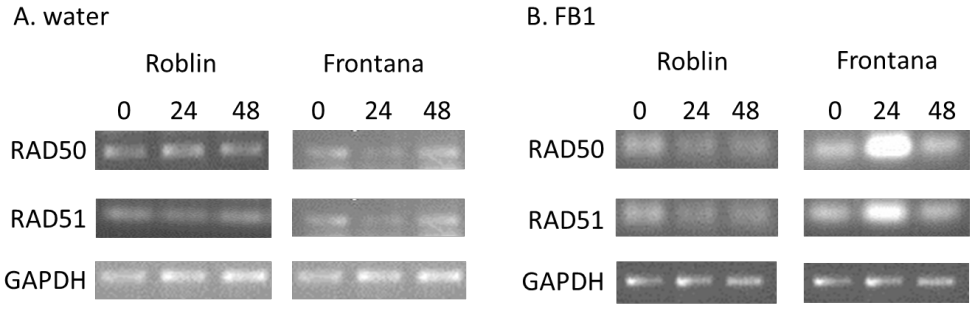

Figure 3. The expression of DNA repair genes. RNA was extracted from wheat leaves treated with water or FB1 for 0, 24 and 48 hrs. **A**. RAD50 and RAD51 gene expression in wheat leaves treated with water (control). **B**. RAD50 and RAD51 gene expression in wheat leaves treated with FB1. GAPDH gene was used as an internal standard. Three experiments were carried out with similar results.

Discussion

Plants mount numerous defense responses to survive pathogen attack. One of these is programmed cell death, which is characterized by the rapid death of plant cells at the site of pathogen infection [LAM, 2004]. Fumonisin B1, a PCD-eliciting fungal toxin, is a sphinganine analogue that has been shown to trigger dosage-dependent cell death in *Arabidopsis* that shares many features with effector-induced cell death [STONE & al. 2000; ASAI & al. 2000]. In this work, we have analyzed FB1-induced cell death in FHB resistant and FHB susceptible wheat cultivars, Frontana and Roblin, respectively. In both cultivars, cell death occurred when leaves were treated with FB1. Cell death occurred earlier in Frontana leaves than in Roblin leaves, suggesting that the particular host-pathogen interaction determines the rapidity of activation of defense responses. This can be explained by the fact that certain levels of disease symptoms in susceptible plants appear over the course of days, whereas defense responses are induced within hours in 'gene-for-gene' immunity [POZO & al. 2004]. Timely activation of cell death is necessary in some host-pathogen interactions for pathogen containment, whereas in other situations cell death is either not essential or not sufficient for disease resistance [LAM & al. 2001]. Our observations have shown more cell death occurred in Roblin leaves than in Frontana leaves after 2 to 3 days treatment with FB1, so it is possible that early activation of host cell death in Frontana would likely limit pathogen proliferation. It should also be noted that upon FB1 treatment the levels of cell death detected by aniline blue assay and by trypan blue assay correlated well, suggesting that the cell death indicated in trypan blue assay is associated with callose deposition, which is triggered by ROS. Therefore, our results suggested that cell death in wheat leaves upon FB1 treatment is associated with concurrent accumulation of ROS.

FB1 induces apoptosis-like PCD in both plants and animals [ASAI & al. 2000]. In *Arabidopsis*, FB1 treatment initiated nuclear DNA fragmentation preceding the loss of membrane integrity, which resembles apoptosis typically associated with PCD in animal cells [STONE & al. 2000]. DNA smear is observed in necrosis, which is the result of severe detrimental changes in the environment of affected cells and is not an active gene-dependent form of cell death [DANON & al. 2000]. Normally, concurrent nuclease and protease activity causes necrosis and a DNA smear on agarose gels [WYLLIE & al. 1980]. FB1 treatments triggered DNA smearing instead of laddering in our current work (Figure 2). It is possible that at certain stages nucleases and proteases are involved in FB1-induced necrosis in wheat leaves. It is also possible that wheat leaves mainly undergo necrosis instead of apoptosis (or both) with FB1 treatment.

Genes that have been identified in wheat that may have a role in DNA repair include RAD50 and RAD51 [LLOYD & al. 2007]. RAD50 was shown to contribute to DNA damage repair, DNA replication, meiosis, and telomere maintenance [CZORNAK & al. 2008; LAMARCHE & al. 2010]. RAD51 was shown to be involved in DNA strand exchange and meiosis [KHOO & al. 2008; SHINOHARA & al. 1992]. It seems possible that DNA repair mechanism may help reduce the damage in Frontana during FB1 treatment. Meanwhile, the mechanisms underlining the observed DNA smearing should be further studied for a better understanding of FB1-induced cell death.

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INCIDENCE AND SEVERITY OF FUNGAL AND COMMON VIRAL DISEASES OF SOME SOYBEAN LINES IN A DERIVED GUINEA SAVANNAH AGRO-ECOLOGY

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Abstract: Nigeria is the largest producer and consumer of soybean in Sub-Saharan Africa with a low yield of less than 1 ton per hectare. Plant diseases play a major role in yield reduction for the crop. The study was to investigate the incidence and severity of fungal and common viral diseases of some soybean lines and determine their effects on soybean yield. Nineteen newly developed soybean lines with two local checks were evaluated. Fungal isolates were identified using cultural and morphological characteristics while Antigen Coated Plate-Enzyme-Linked Immunosorbent Assay was used for detecting viruses. Data were subjected to Analysis of Variance and means were separated at $P \leq 0.05$ using Duncan's Multiple Range Test. Eight fungi isolated from diseased soybean plants were *Fusarium oxysporum*, *Choanephora infundibulifera*, *Colletotrichum gloeosporioides*, *Culvularia* spp., *Fusarium verticilloides*, *Aspergillus flavus*, *Lasiodiplodia theobromae* and *Pestalotia* spp., while the common viral symptom on the field was mosaic and mottling. *F. oxysporum* recorded the highest frequency of occurrence of 40.91% and 22.30%, in both years. *F. oxysporum* and *C. infundibulifera* showed characteristics symptoms of blight when used for pathogenicity on both checks. The soybean lines differed significantly [$P \leq 0.05$] in disease incidence and severity for both fungi and viral diseases. All lines were positive for Cowpea mild mottle virus [CPMMV] in 2016. All the lines evaluated were moderately resistant to leaf blight disease. The study concluded that these lines were tolerant to all observable diseases occasioned by their abilities to produce higher grain yield compared with the local checks, despite the high disease incidence and severity.

Keywords: Cowpea mild mottle virus, *Fusarium* blight, *Fusarium oxysporum*, incidence and severity, leaf blight, resistant.

Introduction

Soybean (*Glycine max* L.) is one of the most important oilseeds crop all over the world [ANONYMOUS, 2018] widely cultivated in tropical, subtropical, and temperate climates of the world [IITA, 2009]. The spread of the crop from its native land of origin has been mainly due to its adaptability and predominant use as a food crop for human nutrition, source of protein for animals, medicinal plant and lately as an industrial crop [YUSUF & IDOWU, 2001]. This legume provides cheap and high-quality protein, containing all amino acids essential for human nutrition when compare to meat and eggs. The crop can be successfully grown in many states of the country, using low agricultural input. Its cultivation

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in Nigeria has expanded as a result of its nutritive and economic importance and diverse domestic usage. Soybean has an average protein content of 40% and is more protein-rich than any of the common vegetable or animal food sources found in Nigeria. When oil is extracted from soybean the residue left is used as protein supplement in livestock feeds. Soybean seeds also contain about 20% oil on a dry matter basis, and this is 85% unsaturated and cholesterol-free [DUGJE & al. 2009].

Nigeria is the largest producer and consumer of soybean in Sub-Saharan Africa with a low yield of less than 1 ton per hectare [IITA, 2009]. Several factors are attributed to this low yield, such as climatic conditions, differences in rainfall patterns, outbreak of diseases and pests etc. Among these factors, plant diseases play a major role in yield reduction for the crop. The increase in the number of soybean diseases and their expansion emanate from intensive production and increased acreage in new regions of the world [HARTMAN & al. 2005]. More than 300 diseases have been reported to affect soybean worldwide [HARTMAN & al. 1999; HARTMAN & al. 2005]. All parts of soybean plant are susceptible to a number of pathogens which reduce quality and/or quantity of seed yield, due to the facts that propagules of various pathogens have increased to densities that cause economic yield losses [HARTMAN & al. 2005].

Among the devastated diseases of soybean are, *Fusarium* blight or wilt disease of soybean, caused by the common soil-borne fungus *Fusarium oxysporum*; it is one of the most destructive diseases of soybean [HASHEM & al. 2009; FAYZALLA & al. 2009]. The pathogen can affect soybeans at any stage of development [FERRANT & CARROLL, 1981]. *F. oxysporum* can also cause root rot and wilt disease of soybean [RAHMAN & al. 2020] *Fusarium* blight symptoms are more noticeable under reduced moisture and hot conditions. The pathogen is difficult to control owing to its persistence nature in the soil and wide host range [ABDEL-MONAIM & al. 2011]. *Phytophthora sojae* causes seedling blight, root and stem rot, this disease is rapidly becoming a very destructive disease in Nigeria [DUGJE & al. 2009]. Asian soybean rust, caused by *Phakopsora pachyrhizi*, is another important soybean foliar disease in Nigeria. The infected leaves have small tan to dark brown or reddish-brown lesions on which small raised pustules occur on the lower surface of the leaves, severe infection leads to premature defoliation and yield losses up to 80% had been recorded [DUGJE & al. 2009]. The crop is also, susceptible to several viruses transmitted by aphids, beetles and whiteflies prevailing in Nigeria, Cowpea mild mottle virus (CPMMV; genus *Carlavirus* transmitted by whitefly (*Bemisia tabaci*) is the most prevalent virus associated with soybean mosaic disease in Nigeria [DUGJE & al. 2009]. Infection with Soybean mosaic virus (SMV) causes mosaic symptoms (light and dark green areas, chlorosis, and leaf curl), necrosis (necrotic areas, stem browning, and stem tip necrosis), and seed mottling, resulting in serious yield losses [ZHENG & al. 2005], yield losses due to SMV infection range from 8% to 50% under natural field conditions [HILL, 1999], to total crop loss during severe outbreaks [LIAO & al. 2002].

Hence, the objectives of this study were, to investigate the incidence and severity of fungal and common viral diseases of some soybean lines, confirm the pathogenicity of the isolates and to determine their effects on soybean yield.

Materials and methods

Experimental site

This study was carried out during the 2015 late cropping season and 2016 early cropping season at Research Farm of National Cereal Research Institutes, Ibadan Research Station, Latitude 7º22' N and Longitude 3º58' E with mean annual rainfall of 1150-1250 mm.

Soybean Lines used

The lines are Early lines: TGx 1990-40F, TGx 1989-48FN, TGx 1989-68FN, TGx 1990-55F, TGx 1989-40F, TGx 1990-52F, TGx 1989-49FN, TGx 1990-57F, TGx 1990-55F, TGx 1485-1D (Check). Medium lines: TGx 1989-45F, TGx 1989-11F, TGx 1989-75FN, TGx 1990-114FN, TGx 1990-78F, TGx 1993-4FN, TGx 1989-53FN, TGx 1990-95F, TGx 1989-42F, TGx 1990-110FN, TGx 1448-2E (Check), and were collected from International Institute for Tropical Agriculture (IITA), Ibadan.

Experimental design and disease assessment

The lines were laid out in a Randomized Complete Block Design with three replications. Plot sizes were 4m row length with inter-row spacing of 50cm and 5cm intra-row, and were observed for natural development of foliar diseases symptoms. Soybean leaf blight severities were determined according to [ABDOU & al. 2001] using rating scale of 1-5:1 = no yellow/spots on leaf, 2 = (1-25%) yellow colouration on one leaf, 3 = (26-50%) yellow colouration on more than one leaf, 4 = (51-75%) yellow colouration plus one wilted leaf, 5 = (76-100%) yellow colouration with more than one wilted leaf. While virus disease severities on the different plots were assessed using a modified scale of 1-5, by ASADI (2005): 1 = no visible symptoms, 2 = mild leaf mottling, 3 = chlorosis and mottling, 4 = stunted with severe mottling and chlorosis, 5 = stunted, severe mottling, leaf bunching, chlorosis with leaf defoliation. Disease incidence was determined by counting diseased plants and expressing it as a percentage of total plants in each plot.

Resistance level

The plants were rated as tolerant, resistant or susceptible on the basis of the following scales: resistance or otherwise to fungal diseases were assessed according to [EL-BRAMAWY & ABD AL-WAHID, 2009], using a scale of 1-5 based on the % disease incidence of: 0.1-20% resistant (R), 20.1-40% moderately resistant (MR), 40.1-50% moderately susceptible (MS), 50.1-75% susceptible (S), 75.1-100% highly susceptible (HS). And soybean viral resistances were assessed based on the mean severity, using modified scale of 1-5 by AKBAR & al. (2015): 1 = (1.0-1.9) highly resistant (H), 2 = (2.0-2.99) moderately resistant (MR), 3 = (3.0-3.99) moderately susceptible (MS), 4 = (4.0-4.99) susceptible (S), 5 = (5 and above) highly susceptible (HS).

Isolation and identification of pathogen associated with soybean foliar diseases

Potato dextrose agar was used for fungal cultures, by dissolving thirty-nine grams of the agar in 1000mls of distilled water and autoclaved at 121°C for 15 minutes, allowed to cool sufficiently before pouring on Petri dishes, 2 mm of the diseased sample, surface sterilized in 3% sodium hypochlorite solution for 1 minute, rinsed in sterile distilled water and then dried in three folds of Whatman's filter paper was then inoculated on the agar aseptically and was incubate at room temperature for 72 hours. The various fungal isolates from each of the samples were sub-cultured to obtain pure cultures for identification. The structural features of colony, colour, extent of growth, presence or absence of mycelia, spores and the nature of colony surface were observed. Microscopic examination involved slide mounts of each isolates and stained with Lacto phenol cotton blue. Fungal identification was confirmed with

the aid of books by BARNETT & HUNTER (1999), ALEXOPOULOS & al. (2002) and AGRIOS (2005).

Antigen coated plate-enzyme-linked immunosorbent assay [ACP-ELISA] for viruses' assay

Leaf samples collected were stored at 4 °C and were tested using ACP-ELISA for the presence of Cowpea Aphid-borne mosaic virus (CabM), Black eye cowpea mosaic virus (BICMV), Cucumber mosaic virus (CM), Soybean mosaic virus (SBMV), Cowpea mottle virus (CpMov), Cowpea yellow mosaic virus (CYMV) and Cowpea mild mottle virus (CPMMV) using homologous rabbit polyclonal antiserum available in the virology unit at IITA Ibadan, following the procedure for ACP-ELISA.

Pathogenicity of the isolated organisms on healthy soybean

All the pathogens isolated from infected soybean leaf were inoculated into healthy soybean plant to determine whether they could induce similar symptoms on re-inoculation. Fungal suspension (ranges from $10^4 – 10^6$ spore /ml) was prepared from the 8 days old culture plates of the isolated fungi. The Mycelia mass of the fungus growth culture in the Petri dishes were scooped out into a sterile conical flask, which contains 10 ml of sterile distilled water, and a drop of Tween 20 detergent (for spore dispersal) was added [TODD, 2022; KEHINDE, 2008]. Inoculated soybean seedlings were covered with a transparent polythene bag for 24 hours to maintain high humidity required for disease initiation and disease symptoms were observed for up to 15 days. Distilled water served as negative control.

Data collection and analyses

Agronomic data taken includes, days to 50% flowering, days to maturity, height at harvest (cm), lodging at harvest, shattering, number of pods/plant, number of seeds/plant, grain yield (kg/ha) and 100 seed weight (g). All the data collected were subjected to analysis of variance (ANOVA), using SAS system 9.1 edition and means values separated and compared using Duncan's Multiple Range Test (DMRT) at 5% significant level of probability.

Results

Pathogens isolated from disease soybean leaf

Eight fungi were isolated from disease soybean plant in this study, *Fusarium oxysporum*, *Culvularia* spp., *Fusarium verticilloides*, *Choanephora infundibulifera* and *Aspergillus flavus* in 2015, while the same fungi were also isolated in 2016, including, *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae* and *Pestalotia* spp.

The Percentage occurrence of pathogens isolated

The percentage occurrence of fungi isolates from disease soybean leaf were presented in Figure 1. *F. oxysporum* had the highest percentage of occurrence in both years (40.91% and 22.3%), followed by *Culvularia* spp. (26.67% and 12.63%), *F. verticilloides* (22.22% and 10.20%), *C. infundibulifera* (5.00% and 17.50%) and *A. flavus* (5.20% and 8.32%). While *C. gloeosporioides* (11.5%), *L. theobromae* (7.30%) and *Pestalotia* spp. (10.25%) were only isolated in 2016.

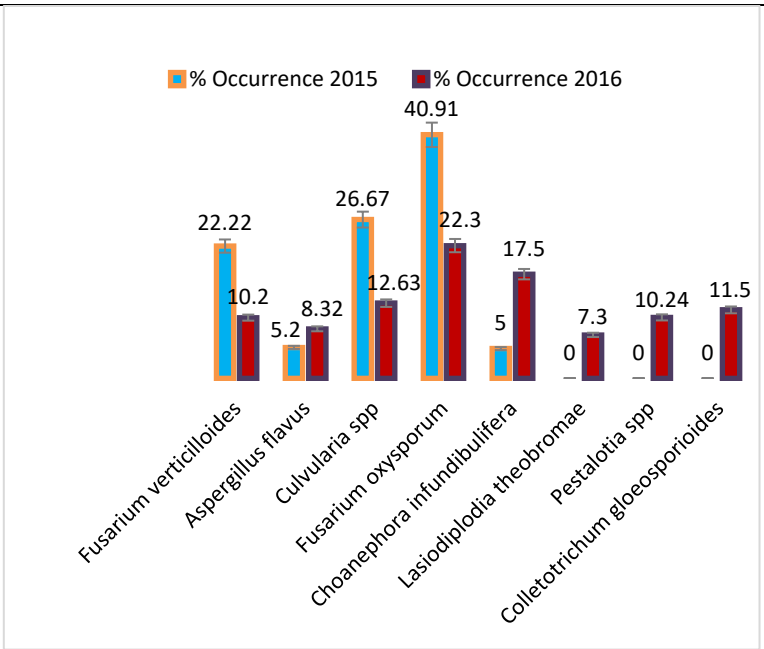

Figure 1. Percentage Occurrence of Fungi isolates from disease soybean leaf

Pathogenicity test

F. oxysporum, showed characteristics symptom of blight when used for pathogenicity on both checks, disease symptoms were first noticed on the lower (older) leaves 7 day after inoculation. The leaves turned yellow and upper leaves of infected plants appear scorched. *C. infundibulifera*, also showed blighted symptoms 7 day after inoculation with grayish patches developed on the leaves and later became necrotic.

Incidence and severity of *Fusarium* blight

Table 1 shows the average disease incidence and severity of *Fusarium* blight in early maturing lines, in 2015 and 2016 planting season, over the period of 10 weeks, after planting. TGx 1485-1D (Check) had significantly ($P \leq 0.05$) higher disease incidence of (47.90%) and (32.33%) in both years respectively, while lines TGx 1990-40F, 1989-48FN, 1989-68FN, 1990-55F and 1989-40F had significantly lower disease incidence than all other lines in 2016. The check also recorded significantly higher disease mean severities of (4.67) and (4.50) than lines TGx1989-49FN and TGx 1990-55F in 2015 and 2016 respectively.

Table 2 shows the average disease incidence and severity of *Fusarium* blight in medium maturing lines, in 2015 and 2016 planting season, over the period of 12 weeks, after planting. TGx1448-2E (Check) had significantly ($P \leq 0.05$) higher disease incidence of (47.50%) than all the lines evaluated in 2015, while the check (36.67%) and TGx 1989-42F (34.17%) had significantly higher disease incidence than other lines in 2016. TGx 1989-45F and TGx 1989-11F recorded significantly ($P \leq 0.05$) lower disease mean severity of (2.50) and (1.50) than the check (3.78) in 2015, while there was no significant difference for disease severity among all the lines evaluated in 2016.

Incidence and severity of *Choanephora* leaf blight

For early maturing lines; in 2015, TGx 1990-40F (16.17%) recorded significantly (P≤ 0.05) lower disease incidence than all the lines evaluated and the check, the same trend was also recorded in 2016 for the same line. The Check (TGx 1485-1D) had significantly (P≤ 0.05) higher mean disease severity of (4.50) and (3.33) than all the lines in both years respectively (Table 1). In the medium maturing lines, TGx1448-2E (Check) and TGx 1989-42F recorded significantly (P ≤ 0.05) higher disease incidence of (36.67%) and (34.17%) respectively than all the lines evaluated in 2015, while the check recorded higher mean severity of (2.64) and (4.36) in both years respectively (Table 2).

Table 1. Average disease Incidence and Severity for *Fusarium and Choanephora* leaf blight in early maturing soybean lines, in 2015 and 2016 planting season

Early Lines	*Fusarium* leaf blight				*Choanephora* leaf blight			
	Incidence		Severity		Incidence		Severity	
	Year's		Year's		Year's		Year's	
	2015	2016	2015	2016	2015	2016	2015	2016
TGx 1990-40F	38.50^{d}	18.90c	4.00abc	3.67bc	16.17f	13.00^{g}	3.00^{c}	2.00^{e}
TGx1989-48FN	35.90^{f}	19.50c	3.50bcd	3.50c	21.50e	16.17^{f}	3.17^{c}	2.17^{de}
TGx1989-68FN	37.33^{e}	20.20c	3.33cd	3.83bc	20.33e	16.83^{f}	3.00^{c}	2.17^{de}
TGx 1990-55F	37.50^{e}	19.00c	4.00abc	3.50c	21.17e	15.50^{fg}	3.00^{c}	2.17^{de}
TGx 1989-40F	38.50^{d}	19.83c	4.17abc	3.83bc	21.00e	16.33^{f}	3.50^{bc}	2.50^{cd}
TGx 1990-52F	36.47^{f}	30.67a	4.00abc	4.00abc	30.67bc	27.16^{cd}	3.50^{bc}	2.50^{cd}
TGx1989-49FN	34.67^{g}	31.00a	2.83d	3.67bc	29.50c	26.00^{de}	3.17^{c}	2.50^{cd}
TGx 1990-57F	43.67^{c}	26.33b	4.50ab	3.87bc	29.33c	27.67^{bcd}	3.70^{b}	2.50^{cd}
TGx 1990-46F	45.50^{b}	32.33a	4.17abc	4.00abc	26.67d	23.83^{e}	3.17^{c}	2.50^{cd}
TGx1485-1D (Check)	47.90^{a}	32.33a	4.67a	4.50a	35.50a	32.00^{a}	4.50^{a}	3.33^{a}

*Means with the same alphabet are not significantly different from each other in the same column (P ≥ 0.05)

Table 2. Average disease incidence and severity for *Fusarium* and *Choanephora* leaf blightin medium maturing soybean lines, in 2015 and 2016 planting season

Medium Lines	*Fusarium* leaf blight				*Choanephora* leaf blight			
	Incidence		Severity		Incidence		Severity	
	Year's		Year's		Year's		Year's	
	2015	2016	2015	2016	2015	2016	2015	2016
TGx 1989-45F	42.00^{f}	22.33^{cd}	2.50bc	3.60^{cde}	22.33^{cd}	24.83^{g}	2.00^{c}	3.00^{ef}
TGx 1989-11F	42.33^{e}	25.17^{c}	1.50c	3.50^{de}	25.17^{c}	25.17^{fg}	2.17^{bc}	3.33^{cde}
TGx1989-75FN	42.01^{f}	22.17^{cd}	3.56ab	3.50^{de}	22.17^{cd}	28.83^{ef}	2.00^{c}	2.83^{ef}
TGx1990-114FN	42.50^{d}	20.83^{d}	3.06ab	3.17^{e}	20.83^{d}	23.83^{g}	2.00^{c}	2.67^{f}
TGx1990-78FN	42.00^{f}	23.00^{cd}	3.50ab	3.33^{de}	23.00^{cd}	28.67^{ef}	2.22^{abc}	3.00^{ef}
TGx 1993-4FN	42.00^{f}	24.67^{c}	3.17ab	3.33^{de}	24.67^{c}	31.00^{de}	2.46^{ab}	3.33^{cde}
TGx1989-53FN	46.01^{b}	20.33^{d}	3.56ab	3.83^{cd}	20.33^{d}	25.17^{fg}	2.15^{bc}	3.17^{def}
TGx 1990-95F	44.00^{c}	22.33^{cd}	3.65ab	3.83^{cd}	22.33^{cd}	27.33^{fg}	2.17^{bc}	3.17^{def}
TGx 1989-42F	42.50^{d}	34.17^{a}	3.17ab	3.67^{cde}	34.17^{a}	36.17^{bc}	2.33^{abc}	3.56^{bcd}
TGx1990-110FN	42.00^{f}	23.37^{cd}	3.45ab	3.66^{cde}	23.37^{cd}	25.67^{fg}	2.17^{bc}	3.17^{def}
TGx1448-2E (Check)	47.50^{a}	36.67^{a}	3.78a	4.70^{a}	36.67^{a}	43.33^{a}	2.64^{a}	4.36^{a}

*Means with the same alphabet are not significantly different from each other in the same column (P ≥ 0.05)

Incidence and severity of virus diseases

Table 3 shows the average incidence and severity of virus diseases in early maturing soybean lines, in 2015 and 2016 planting season, over the period of 10 weeks after planting. TGx 1990-40F and TGx 1989-48FN recorded significantly ($P \leq 0.05$) lower disease incidence of (36.67%) and (36.83%) respectively, than TGx 1990-46F (43.37%); they also recorded significantly disease mean severity of (2.06) and (2.50) than TGx 1990-46F (3.96) in 2015. TGx 1990-40F had significantly average disease incidence of (17.34%) and also recorded lower mean disease severity than the check in 2016.

The average incidence and severity of virus diseases in medium maturing soybean lines, in 2015 and 2016 planting season, over the period of 12 weeks after planting, was presented in Table 4; TGx 1989-11F had significantly ($P \leq 0.05$) lower disease incidence of (37.83%) and mean disease severity of (1.37), than all the lines and the check in 2015. The check also recorded significantly higher disease incidence of (48.33%) than all the lines evaluated in 2016.

Virus assayed

Serological – Incidence of Soybean virus assayed in leaf sample of early and medium maturing soybean lines in 2015 and 2016 planting season were presented in Tables 3 and 4 respectively. In 2015, all early maturing lines tested negative for all the viruses assayed, except TGx 1989-68FN that tested positive for Cowpea mild mottle virus (CPMMV). All lines were positive for Cowpea mild mottle virus (CPMMV) in 2016, while the Check tested positive for Cowpea mottle virus (CpMov) in same year. In medium maturing lines, all lines were negative for all the virus assayed in 2015 and 2016, but positive for Cowpea mild mottle virus (CPMMV) in 2016.

Table 3. Average disease Incidence and Severity for Virus in early maturing soybean lines, in 2015 and 2016 planting season

Early Lines					Serology – Incidence of Soybean Virus Assayed in Leaf Sample using ACP-ELISA					
	2015		2016		2015			2016		
	Incidence	Severity	Incidence	Severity	CpMoV	CYMV	CPMMV	CpMoV	CYMV	CPMMV
TGx 1990-40F	36.67^{e}	2.06^{d}	17.34^{e}	2.97^{c}	–	–	–	–	–	+
TGx 1989-48FN	36.83^{e}	2.50^{d}	28.40^{a}	3.07^{bc}	–	–	–	–	–	+
TGx 1989-68FN	39.22^{d}	3.04^{c}	28.37^{a}	3.10^{bc}	–	–	+	–	–	+
TGx 1990-55F	39.78^{c}	3.13^{c}	22.94^{cd}	3.19^{abc}	–	–	–	–	–	+
TGx 1989-40F	38.81^{d}	3.52^{abc}	20.65^{d}	3.38^{abc}	–	–	–	–	–	+
TGx 1990-52F	38.96^{d}	3.37^{bc}	28.33^{a}	3.12^{bc}	–	–	–	–	–	+
TGx 1989-49FN	39.21^{d}	3.18^{bc}	27.00^{ab}	3.36^{abc}	–	–	–	–	–	+
TGx 1990-57F	42.22^{b}	3.76^{ab}	25.72^{abc}	3.66^{ab}	–	–	–	–	–	+
TGx 1990-46F	43.37^{a}	3.96^{a}	24.65^{bc}	3.31^{abc}	–	–	–	–	–	+
TGx 1485-1D (Check)	39.94^{c}	3.37^{bc}	28.34^{a}	3.79^{a}	–	–	–	+	–	+

Table 4. Average disease Incidence and Severity for Virus in Medium Maturing Soybean lines, in 2015 and 2016 planting season

Medium Lines					Serology – Incidence of soybean virus assayed in leaf Sample using ACP-ELISA					
	2015		2016		2015			2016		
	Incidence	Severity	Incidence	Severity	CpMoV	CYMV	CPMMV	CpMoV	CYMV	CPMMV
TGx 1989-45F	43.01^{h}	2.63^{b}	21.50^{f}	2.10^{b}	–	–	–	–	–	+
TGx 1989-11F	37.83^{k}	1.37^{c}	26.80^{bcde}	2.40^{ab}	–	–	–	–	–	+
TGx1989-75FN	42.22^{i}	2.50^{b}	24.37^{ef}	2.15^{ab}	–	–	–	–	–	+
TGx1990-114FN	44.33^{f}	2.48^{b}	30.23^{bc}	2.33^{ab}	–	–	–	–	–	+
TGx1990-78FN	41.51^{j}	2.63^{b}	25.67^{cdef}	2.11^{b}	–	–	–	–	–	+
TGx 1993-4FN	43.72^{g}	3.13^{ab}	28.73^{bcde}	2.40^{ab}	–	–	–	–	–	+
TGx1989-53FN	45.17^{c}	3.15^{ab}	24.91^{def}	2.30^{ab}	–	–	–	–	–	+
TGx 1990-95F	51.67^{a}	3.70^{a}	31.05^{b}	2.49^{a}	–	–	–	–	–	+
TGx 1989-42F	44.84^{d}	3.41^{ab}	30.53^{b}	2.19^{ab}	–	–	–	–	–	+
TGx1990-110FN	44.50^{e}	2.98^{ab}	25.50^{cdef}	2.03^{b}	–	–	–	–	–	+
TGx1448-2E (Check)	47.54^{b}	3.37^{ab}	48.33^{a}	2.29^{ab}	–	–	–	–	–	+

*Means with the same alphabet are not significantly different from each other in the same column ($P \geq 0.05$). Virus assayed by antigen coated-plate enzyme-linked immunorbent assay (ACP-ELISA); **CpMov**, Cowpea mottle virus; **CYMV**, Cowpea yellow mosaic virus; **CPMMV**, Cowpea mild mottle virus

Grain yield of soybean and resistance level to the diseases

Table 5 shows grain yield in kilogram per hectare and resistance level in early and medium maturing soybean lines, in 2015 and 2016 planting season. In early maturing lines, TGx 1989-40F (294.07 kg/ha) and TGx 1989-49FN (264.43 kg/ha) recorded significant ($P \geq 0.05$) lower grain yield than the Check (567.40 kg/ha) and TGx 1990-46F (465.97 kg/ha). The same trend was also observed for these lines in 2016. There was no significant difference in grain yield for both years in medium maturing lines.

All the lines evaluated were moderately resistant to leaf blight disease for both the maturing groups. While lines: TGx 1990-40F, TGx 1989-48FN, TGx 1989-68FN, TGx 1990-55F, TGx 1990-52F and TGx 1989-49FN are highly resistant to the virus diseases in early maturing lines; TGx 1993-4FN, TGx1989-53FN, TGx 1990-95F, TGx 1989-42F and TGx1990-110FN were moderately resistant to the same disease in medium maturing lines. The checks were moderately susceptible to all the diseases evaluated in this study in both maturity groups (Table 5).

Table 5. Grain yield and resistance level in early and medium maturing soybean lines 2015 and 2016 planting season

Early Lines	Grain yield (kg/ha)		Resistance Level		Medium Lines	Grain yield (kg/ha)		Resistance Level	
	2015	2016	*Fusarium* blight/wilt	Viral disease		2015	2016	*Fusarium* blight/wilt	Viral disease
TGx 1990-40F	345.93^{bc}	547.80^{bc}	MR	HR	TGx 1989-45F	370.40^{a}	589.70^{a}	MR	HR
TGx 1989-48FN	346.67^{bc}	563.70^{bc}	MR	HR	TGx 1989-11F	436.30^{a}	657.00^{a}	MR	HR
TGx 1989-68FN	342.20^{bc}	559.40^{bc}	MR	HR	TGx1989-75FN	288.90^{a}	509.60^{a}	MR	HR
TGx 1990-55F	402.97^{bc}	634.37^{bc}	MR	HR	TGx1990-114FN	303.70^{a}	532.00^{a}	MR	HR
TGx 1989-40F	294.07^{c}	589.83^{c}	MR	MR	TGx1990-78FN	251.90^{a}	472.70^{a}	MR	HR
TGx 1990-52F	397.03^{bc}	627.07^{bc}	MR	HR	TGx 1993-4FN	340.50^{a}	556.70^{a}	MR	MR
TGx 1989-49FN	264.43^{c}	523.00^{c}	MR	HR	TGx1989-53FN	389.60^{a}	603.40^{a}	MR	MR
TGx 1990-57F	307.43^{bc}	540.57^{bc}	MR	MR	TGx 1990-95F	403.00^{a}	621.20^{a}	MR	MR
TGx 1990-46F	465.97^{ab}	696.30^{ab}	MR	MR	TGx 1989-42F	272.60^{a}	696.80^{a}	MR	MR
TGx1485-1D (Check)	567.40^{a}	794.43^{a}	MS	MS	TGx1990-110FN	472.60^{a}	603.70^{a}	MR	MR
					TGx1448-2E (Check)	486.70^{a}	733.20^{a}	MS	MS

*Means with the same alphabet are not significantly different from each other in the same column ($P \geq 0.05$)
R= Resistant; HR= Highly Resistant; MR= Moderately Resistant; MS= Moderately Susceptible.

Discussion

Eight fungi were isolated from disease soybean plant in this study, from the pathogenicity test, only *F. oxysporum* and *C. infundibulifera* were the pathogenic ones. This result agrees with the findings of previous researchers who had associated these organisms with soybean foliar disease [FAYZALLA & al. 2009; HASHEM & al. 2009; SUBBA RAO & al. 1990]. While other fungal isolates shown no know symptom(s) when used for pathogenicity on any of the soybean checks either singly or mixed, except for *C. gloeosporioides* and *C. infundibulifera* that was re-isolated when mixed with *F. oxysporum*, they could be secondary invaders or opportunist pathogens.

F. oxysporium had the highest percentage of occurrence in both maturing lines. This pathogen can cause blight or wilt disease in soybean, and has been reported as one of the most destructive diseases of soybean [HASHEM & al. 2009; FAYZALLA & al. 2009], the pathogen can affect soybeans at any stage of development [FERRANT & CARROLL, 1981]. According to NELSON & al. (1997) and YANG (1997), *Fusarium* species are often favoured by cool temperatures, particularly in the early growing season. The decreased in moisture condition of the soil during the 2015 growing period in this study could have triggered the susceptibility of the crop to *Fusarium* blight. This result corroborates the findings of ZHANG & al. (2010) who concluded that as soil moisture becomes more limiting, soybeans become stressed, thereby increasing susceptibility to infection by *Fusarium*. DAS & al. (2019) also reported that plant infection by *Fusarium* can occur from seeds germination to mature stage, depending on the host and *Fusarium* species. *Choanephora* Leaf blight caused by fungus *C.*

infundibulifera Sacco. was also isolated in this study, as one of the foliar diseases of soybean, SUBBA RAO & al. (1990) have also documented the pathogen during their study of stem canker pathogen on soybean.

The variation in the disease incidence and severity observed in the lines evaluated, in both years could be attributed to differences in resistance status of each line and to the difference level of virulence in the pathogen. ODUBANWO & al. (2013) was also of the opinion that, soybean resistance depends on the lines level of expression and to their ability over time to tolerate the attack of the pathogens. Symptom of *fusarium* blight was more pronounced at about 6 weeks after planting, disease symptoms are first noticed on the lower (older) leaves. The leaves turn yellow and as the disease progresses, the upper leaves of infected plants wilt and appear scorched, in severe cases, the leaves dry up and drop prematurely leaving the petiole behind, which is in conformity with the report of ABIODUN & al. (2016) and NELSON & al. (1997). *C. infundibulifera* incidence was higher in 2016, there was heavy and frequent rainfall in this period, which agrees with SUBBA RAO & al. (1990) who concluded that heavy rainfall is one of the factors responsible for survival and spread of this pathogen.

Virus symptoms from this study majorly are mottling and mosaic symptoms, although other symptoms such as yellow vein banding, necrotic spots and chlorosis were also present in the field. Viruses assayed by antigen coated-plate enzyme-linked immunorbent assay (ACP-ELISA) in the laboratory were not in conformity with the field evaluation for both soybean lines in both years. This was possible because the observations in the field were based solely on visual virus-like symptoms, which were probably caused by other pathogens, physiological disorders and unidentified viruses; this finding gives credence to earlier reports of NJUKENG & al. (2013) that out of the 360 leaf samples of pepper showing virus-like symptoms collected from the field during survey followed by laboratory diagnosis using DAS-ELISA, 117 leaf samples were negative for viruses assayed. Cowpea mild mottle virus (CPMMV) is the commonest virus associated with all soybean lines used in this study, this result agrees with the conclusion of DUGJE & al. (2009) that CPMMV transmitted by whitefly (*Bemisia tabaci*) is the most prevalent virus associated with soybean mosaic disease in Nigeria.

Grain yield is considered an important indicator for any foliar disease. Grain yields among the evaluated lines varied during the two years of this study. TGx1990-46F (early maturing) and TGx1990-110FN (medium maturing) had high grain yield in both years respectively. This agrees with reports from earlier researchers who reported significant yield differences among soybean genotypes [ZHANG & ZHANG, 2000; ABLETT & al. 2000]. These lines were moderately resistant to all the diseases observed on the field, as they were able to produce high grain yield when compared with the local checks, despite the high disease incidence and severity.

Conclusion and recommendation

Leaf blight disease were the foliar diseases found to be associated with soybean lines used in this study and ecology, while Cowpea mild mottle virus (CPMMV) is the commonest virus associated with all soybean lines used. These diseases can reduce grain yield and yield traits, but it depends on the disease's severity and the genetic make-up of each soybean genotypes, these soybean lines could be utilized as parent lines for breeding against soybean foliar diseases and useful for farmers in area endemic to any of the foliar disease encounter in

this study. It is therefore recommended that further studies should be carried out on these soybean lines in other agro-ecological zone to determine the effectiveness of their resistance to foliar diseases as claimed from this study.

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THE GROWTH, DEVELOPMENT AND USE OF NEW TAXA OF THE GENUS *WEIGELA* THUNB. IN GREEN SPACE DESIGN IN THE REPUBLIC OF MOLDOVA

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Abstract: The peculiarities of growth, development and use of 5 new taxa of the genus *Weigela* Thunb. have been described. The studied taxa – *Weigela florida* 'Alexandra', 'Carlton', 'Caricature', 'Minor Black' and 'Pink Princess' – differ in size, crown diameter, annual growth, density of flowering stalks per shoot from the previous year, flower density per 20-cm-long shoot, abundance of flowering, colour of flowers and leaves. All of them can be used successfully in landscaping in the Republic of Moldova.

Keywords: development, introduction, taxa, use, *Weigela* Thunb.

Introduction

The introduction and ecobiological research on the new taxa of woody plants will contribute to the modernization and diversification of their range in green space design in the Republic of Moldova. The new taxa, as well as species of the genus *Weigela* Thunb. that have been recently introduced in our country are plants of high ornamental value, which stand out due to the specific foliage of various shades, the abundance and duration of the flowering, the multitude of shades of the petals and the habit of the shrubs. The genus *Weigela* Thunb., of the family Caprifoliaceae Juss., includes 15 species, widespread in East Asia [PALANCEAN & COMĂNICI, 2009]. In the territory of the former USSR, 3 species occur in the wild and 9 species and a large number of varieties are cultivated [Derevia i custarnichi SSSR, 1962]. In Europe, there are 3 species and many cultivars, which differ in habit, foliage and flower colour. Implementing the Global Biodiversity Conservation Strategy, we have set ourselves the goal of mobilizing and diversifying the *Weigela* Thunb. gene pool. In the "Alexandru Ciubotaru" National Botanical Garden (Institute) (hereinafter NBGI), two species and several cultivars have been introduced recently [ROȘCA & al. 2021]. In Moldova, most of the planting material of ornamental species has been brought from Europe, then researched, acclimatized and used in landscaping different types of green spaces designed as areas for passive and active rest.

The goal of this research was to determine and describe the peculiarities of growth, development and use of 5 new taxa of *Weigela florida*, namely: 'Alexandra', 'Carlton', 'Caricature', 'Minor Black' and 'Pink Princess', under the pedoclimatic conditions of the Republic of Moldova.

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Material and methods

The new taxa of *Weigela florida* – 'Alexandra', 'Carlton', 'Caricature', 'Minor Black', 'Pink Princess' – were introduced in the autumn of 2018, in the nursery of the Dendrology Laboratory of NBGI.

The research was carried out in the introduction nursery of the Dendrology Laboratory as part of the research project 20.80009.7007.19 „Introduction and elaboration of technologies of multiplication and cultivation by conventional techniques and tissue culture of new woody plant species". Five new taxa of Weigela in the third and fourth growing seasons, which grow in the collection of NBGI, have served as research subjects. Morphological parameters were determined in 10 plants, 100 flowers and shoots. The phenological observations were made according to the methodology [ILIESCU, 2002; Metodica fenologhiceschih nabliodenii v botaniceschih sadah SSSR, 1979] that was perfected by Dr. hab. A. Palancean [PALANCEAN & COMĂNICI, 2009]. The landscaping was carried out according to the principles described in the monograph written by the researchers of the University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca [DUMITRAȘ & al. 2008].

Results and discussions

Weigela florida (Bge.) A. DC. is native to northern China and Korea. It is an erect shrub, growing about 2-3 m tall. The shoots have 2 rows of hairs. The leaves are elliptical to oblong-ovate, sometimes obovate, acute, serrate, 5-10 cm long, short petiolate, pubescent on the midrib, on the underside – pubescent on all veins. The leaves develop in April-May, change colour in September-October and fall in October. The flowers with fused sepals are mostly arranged at the top of the plant. The corolla is funnel-shaped, 2.5-3 cm long, with rounded petals, patent, uneven, of different shades from bright red to orange, depending on the biotype. The ovary is pubescent and the stigma bilobed. The shrub is of high ornamental value due to the abundance of flowering and the long flowering period. It blooms in May-June; sometimes repeated flowering takes place in July-August. The fruit is a capsule with 2 valves; their maturation takes place in August-October depending on the weather conditions during the process of ontomorphogenesis of the fruit.

The shrub is resistant to frost, dust, toxins, withstands urban conditions, it is sometimes affected by prolonged heat (leaves partially fall and the abundance of flowering decreases) if the cultivation techniques are not observed. It grows well on rich, light soils, requires minimal care, only loosening the soil around the plant and regular trims to maintain its shape and decorativeness for a longer period.

It is recommended to cultivate *W. florida* plants in the foreground, alone or in small groups, at the edges of woody plantations in most areas of the Republic of Moldova.

Weigela x *hybrida* Jaeg. is a shrub of hybrid origin commonly grown by amateurs in private gardens. It is characterised by abundant and colourful pink, red, purple or white flowers, as well as by green, purple, motley foliage. It is recommended for parks and gardens, in sunny places, since the pastel shades can please the eye and cheer up visitors.

Weigela florida 'Alexandra' – is a shrub with a moderate growth, on average 25 cm per year (Figure 3), with a dense and neat crown. The leaves are lanceolate, dark bronze-purple. The bright pink, funnel-shaped, short petiolate flowers are arranged at the base of the leaves (Figure 1a).

It grows well in full sun and does not need additional irrigation. It tolerates well winter conditions, without additional protection. It is an ornamental shrub of regular and compact shape. The shrub is particularly showy in May-June, when it is abundantly covered with flowers. The density of flowers per shoot is on average 12 pcs. on 20 cm (Figure 3). Due to the colourful foliage, the plant maintains its ornamental value even after the flowering stage. This shrub can be planted either alone or in small groups of the same species or mixed with other species with lighter foliage. This cultivar is particularly impressive if used to create fences, which can delight the visitors and evoke positive emotions.

Weigela florida 'Carlton' – this shrub is a special one due to its foliage, which starts as light green and then turns into a radiant yellow-green. The growth of annual shoots is quite intense (45 cm) and abundant (Figure 3). It grows branches down to ground level, forming a compact hemisphere, and among the bright foliage, flowers of an extremely showy red-ruby colour appear (Figure 1b). The flowers are funnel-shaped, with white anthers, as long as the petals, and bloom in May-June for about a month. This taxon repeatedly blooms in August (Figure 4). The density of flowers per 20-cm-long shoot reaches high values (35 pcs.), as compared with other taxa. It is recommended to plant this cultivar in full sun to obtain a higher abundance of flowers. It is an extremely decorative shrub with exquisite foliage and brightly coloured flowers. It is ideal as a bright accent when forming a group or hedge, but can also be planted alone.

Weigela florida 'Caricature' – is a faster growing shrub (55 cm) as compared with other taxa (Figure 3), with spherical crown with slightly curved shoots. The height of the bush is almost equal to its diameter. The most noteworthy characteristics of this shrub are the raw-green leaves with a cream-white edge, slightly domed in the middle with a somewhat wrinkled appearance. The flowering is very fine, with trumpet-shaped, short-petiolate flowers of several shades of pink. On some shoots, there are pale pink flowers, and on others – more intense pink (Figure 1c). Their number is non-essential, but the appearance is very fine during May-June. The density of flowers per 20-cm-long shoot is lower (10 pcs.) as compared with other taxa (Figure 3). This taxon is particularly showy, attracting all eyes due to its habit and phenotypic variability. Having a fairly long period of decorativeness, it is recommended to plant this cultivar in the foreground when forming mixed or pure groups, but, it is also very attractive solitarily.

Weigela florida 'Minor Black' – dwarf shrub with significantly slower annual growth (20 cm) as compared with other taxa (Figure 3). It has purple-brown foliage that draws attention even if it is a smaller shrub. The leaves maintain their colour throughout the growing season, and the flowering increases its aesthetic value in a green space. The trumpet-shaped, bright pink flowers last for about a month (Figure 2a). According to the density of flowers per shoot (14 pcs.), this taxon occupies an intermediate position, but closer to the cultivar "Alexandra". Being a small shrub, it has multiple recommendations, for example, it is an excellent choice when forming small groups in the foreground, or in small, alpine gardens. It is recommended for container culture, being planted in the middle, surrounded by hanging plants.

Weigela florida 'Pink Princess' – it is one of the most compact-looking taxa. The pale-pink, funnel-shaped, short-petiolate flowers cover the upper part of the shrub (Figure 2b). It blooms in June. A specific feature of this taxon, similar to the 'Carlton' cultivar, is the repeated flowering in August (Figure 4).

The average density of flowers per 20-cm-long shoot is 19 pcs. The light green foliage improves the ornamental value throughout the growing season. The annual growth was quite fast (50 cm) as compared with other taxa (Figure 3).

This shrub is recommended for sunny places, tolerates and partial shade, is generally undemanding to the soil, but requires a medium drained and moist soil. Any care work is done immediately after flowering, because the shrub blooms on the shoots of the previous year. Its neat and fine appearance allows it to be planted alone or in pure groups. Because the fruits and autumn foliage are less attractive, this cultivar should be accompanied by other species to extend the period of decorativeness. It withstands the winter conditions in our country without major damage, sometimes only the tops of the annual shoots can be slightly affected (Figure 5a, b, c).

Figure 1. Taxa in the flowering stage in the third growing season.
a. *Weigela florida* 'Alexandra'; **b.** *Weigela florida* 'Carlton'; **c.** *Weigela florida* 'Caricature'

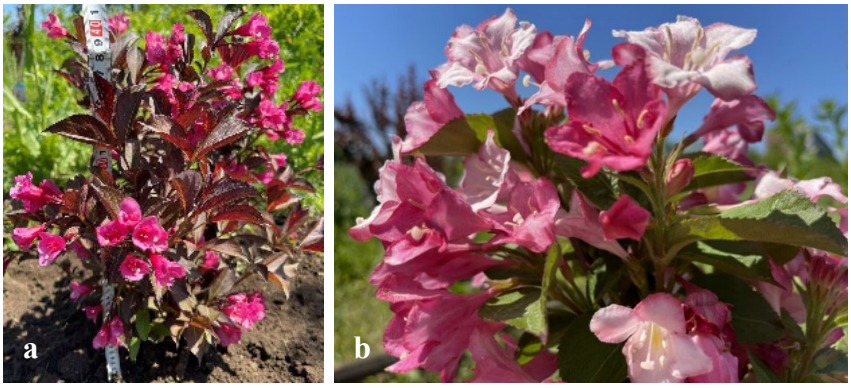

Figure 2. Taxa in the flowering stage in the third growing season.
a. *Weigela florida* 'Minor Black'; **b.** *Weigela florida* 'Pink Princess'

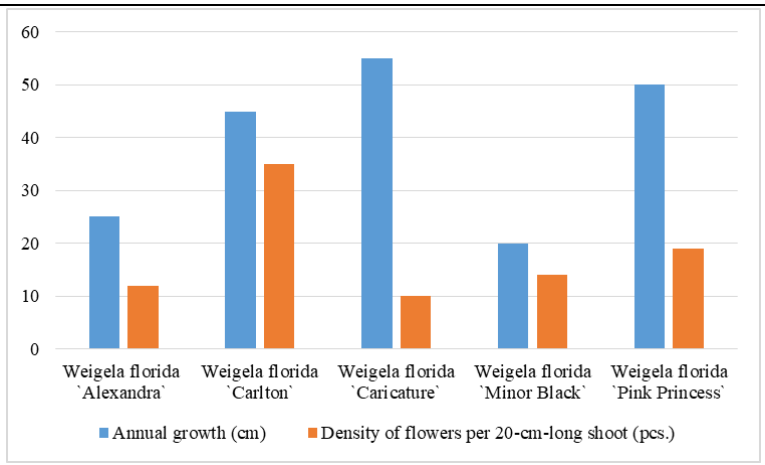

Figure 3. Peculiarities of annual growth and flower density of *Weigela* Thunb. taxa

Luna / decada	IV			V			VI			VII			VIII			IX			X			XI	
Cultivarul	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2
Weigela florida `Alexandra`																							
Weigela florida `Carlton`																							
Weigela florida `Caricature`																							
Weigela florida `Minor Black`																							
Weigela florida `Pink Princess`																							

Figure 4. The phenological spectrum of taxa of the genus *Weigela* Thunb.

Figure 5. The use of *Weigela* Thunb. taxa in green spaces in contrast to other species

Conclusions

The researched taxa of the genus *Weigela* Thunb. ('Alexandra', 'Carlton', 'Caricature', 'Minor Black' and 'Pink Princess') differ in plant height, crown diameter, annual growth, density of flowering stalks per shoot from the previous year, flower density per 20-cm-long shoot, abundance of flowering, length of the flowering period, colour of flowers and leaves throughout the growing season.

The introduced taxa are resistant to the pedoclimatic conditions of the Republic of Moldova, do not require special care, only basic care works and pruning to maintain permanent decorativeness, they can diversify and complete the range of ornamental plants for green spaces in cities and rural areas.

Because the studied *Weigela* Thunb. plants maintain their decorative qualities for a long period, they are recommended for planting in the foreground, in pure groups or mixed with other species of ornamental shrubs. The studied taxa are excellent ornamental shrubs, which can be used in all the regions of the Republic of Moldova.

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GYPSOPHILA × *MOLDAVICA* PÎNZARU NOTHOSP. NOVA IN THE REPUBLIC OF MOLDOVA

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Abstract: The article describes a new hybrid namely *Gypsophila* × *moldavica* Pînzaru (*G. glomerata* × *G. collina*), formed under *ex situ* conditions, in the author's private garden. Arguments have been brought to support the claim of recognizing the species *G. glomerata* Adams and it is considered appropriate to synonymize the species *G. pallasii* Ikonn.

Key words: Caryophyllaceae, distribution, *Gypsophila* × *moldavica* Pînzaru nothosp. nova, morphology, Republic of Moldova.

Introduction

The genus *Gypsophila* L. in the *Flora of Bessarabia* is represented by 8 species, and in the Republic of Moldova – by the following taxa: *G. collina* Steven ex Ser., *G. paniculata* L., *G. perfoliata* L., *G. elegans* M. Bieb. and *G. pallasii* Ikonn. [IZVERSCAIA, 2016]. The species *Gypsophila pallasii* Ikonn. is a name that has not been recognized by all the botanists [GHEIDEMAN, 1986; NEGRU, 2007; SEREGIN, 2008; PÎNZARU & SÎRBU, 2016]. The recent floristic research conducted by the author has brought arguments in favour of the opportune synonymization of the species *G. pallasii* Ikonn. As a result of growing together the species *G. glomerata* Adams and *G. collina* Ser., a new hybrid species appeared in the author's private garden; its detailed description is presented in this article.

Materials and methods

The article is based on the floristic research carried out in natural habitats in the Republic of Moldova, Romania (Constanța county: Cotul Văii village in Albești commune and Tulcea county: nature reserve Capul Doloșman in Jurilovca commune) and Bulgaria (Dobrich province: Topola village in Kavarna municipality), as well as on plants grown under *ex situ* conditions in 2018-2022. The identification of the species was carried out with the help of guides for species identification and already published information regarding the studied species, taking into account the morphological and ecological peculiarities. The exsiccatae are stored in the Herbarium of the "Alexandru Ciubotaru" National Botanical Garden (Instiute) in Chișinău [CHGB], in the Herbarium of "Anastasie Fătu" Botanical Garden of Iași [IAGB], and in the Herbarium of the botanist Pavel Pînzaru at the Tiraspol State University (based in Chișinău) [CHUST-PP].

The dried specimens (exsiccatae) from the Herbarium of "Alexandru Ciubotaru" National Botanical Garden (Institute) were analysed.

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The list of exsiccatae of *Gypsophila glomerata* Adams analysed by the author:

Republic of Moldova:

- Chișinău muncipality, Codru commune, in the author's private garden, 27.07.2017, P. Pînzaru [CHUST-PP 4331], 24.07.2022, P. Pînzaru [IAGB 47744];
- Vulcănești district, Musaid commune, steppe slope, 04.07.1950, leg. L. Nikolaeva; Vulcănești commune, hill with steppe vegetation, 05.07.1950, L. Nikolaeva [CHGB 32302, 32303, 32304], 27.07.1958, L. Nikolaeva [CHGB 32309], 27.07.1958, T. Gheideman [CHGB 32305], 10.07.1986, T. Bogoutdinova [224506]; Etulia commune, steppe slope, 27.07.1958, L. Nikolaeva [CHGB 32310], 27.07.1958, T. Gheideman [CHGB 32308], 06.07.2017, P. Pînzaru [CHUST-PP 4900];
- Taraclia district, Ciumai village in Vinogradovca commune, steppe slope, 26.08.1958, L. Nikolaeva [CHGB32307], 26.08.1958, T. Gheideman [CHGB 32306, 32311]; 09.08.1988, Gh. Postolache [CHGB 221601, 221611, 221612, 221613], 23.08.2009, P. Pînzaru [CHUST-PP 4892];
- Cahul district, Colibaș commune, hill with steppe vegetation, 03.09.1971, A. Istrati [CHGB 32300]; Giurgiulești commune x Cîșlița-Prut commune, hills with loamy soil and steppe vegetation, 19.07.1979, L. Nikolaeva [CHGB 217166, 217167, 217168, 217169]; Slobozia Mare commune, hills with loamy soil and steppe vegetation, 06.08.1997, P. Pînzaru [CHGB 233809], 13.09.2009, V. Ghendov, G. Șabanova, T. Izverscaia (*G. pallasii* Ikonn.), teste T. Izverscaia [CHGB 236773, 236774], 04.09.2013, V. Ghendov, T. Izverscaia (*G. pallasii*) [CHGB 238695], 11.07.2019, P. Pînzaru, V. Cantemir, Ș. Manic [CHGB 239707]; Cîșlița-Prut commune, steppe slope, 12.07.2019, P. Pînzaru [CHUST-PP 4899]; Brînza commune, slope with clayey-sandy soil, 12.07.2019, V. Cantemir, P. Pînzaru [CHGB 239708, 239709]; Văleni commune, slope with steppe vegetation, 11.07.2008, V. Ghendov, T. Izverscaia, G. Șabanova (*G. pallasii*) [CHGB 236320, 236321, 236322, 236323, 236324].

Russia:

- Reg. Stavropol, in the vicinity of Pyatigorsk city, Mashuk Mountain, on limestone soil, 30.06.1901, Iv. Širaevskij [CHGB 215384, 127724].

Bulgaria:

- Dobrich province: Topola village in Kavarna municipality, on limestone hills, 26.08.2022, P. Pînzaru [CHUST-PP 4841, 4842; CHGB 239993; IAGB 47745].

Romania:

- Constanța county: Cotul Văii village in Albești commune, on limestone soil, col. P. Pînzaru, Dan Pînzaru, det. P. Pînzaru, 16.10.2022 [CHUST-PP 4926, 4950];
- Tulcea county: nature reserve Capul Doloșman in Jurilovca commune, on limestone soil, col. P. Pînzaru, Dan Pînzaru, det. P. Pînzaru, 16.10.2022 [CHUST-PP 4960, 4963].

The list of exsiccatae of *Gypsophila collina* Ser., collected by the author:

- Florești district, Gvosdova village in Gura Camencii commune, on limestone hills, 22.07.2017, P. Pînzaru [CHUST-PP 4897]; Stîrceni village in Vărvăreuca commune, in rock fissures, 21.08.2016, P. Pînzaru [CHGB 239540], 23.07.2022, P. Pînzaru [CHUST-PP 4894; CHGB]; Vărvăreuca commune, on limestone hills, 23.07.2022, P. Pînzaru [CHUST-PP 4329]; Cașunca commune, in rock fissures, 17.07.2009, P. Pînzaru [CHUST-PP 4326]; Cenușa village in Roșietici commune, on limestone hills, 21.08.2016, P. Pînzaru

[CHGB 239541]; Ciutulești commune, on limestone hills, 24.07.1947, A. Ivankov, tested by V. Andreev [CHGB 32292];

- Rezina district, Țipova village in Lalova commune, on limestone hills, 15.07.2020, P. Pînzaru [CHGB 239706], 28.07.2021, P. Pînzaru [CHUST-PP 4898];
- Orhei district, Butuceni village in Trebujeni commune, on limestone hills, 04.08.1954, L. Nikolaeva [CHGB 32296, 32297], 22.09.1962, L. Nikolaeva, tested by T. Gheideman [CHGB 32287, 32288, 32289], 22.07.2022, P. Pînzaru [CHUST-PP 4893];
- Criuleni district, Mașcăuți commune, in rock fissures, 22.07.2022, P. Pînzaru [CHUST-PP 4889; CHGB 239992; IAGB 47741];
- Camenca district, Camenca commune, on limestone hills, 26.07.1947, A. Ivankov, tested by A. Andreev [CHGB 32294];
- Dubăsari district, Doibani commune, on limestone hills, Iagorlîc, 07.1934, Kleopov [CHGB 32290], Dubăsari commune, on East-facing slope, col. L. Nikolaeva, 06.08.1950, tested by T. Gheideman [CHGB 32285, 32286, 32295]; Goian commune, on limestone hills, 07.07.1989, P. Popescu, tested by V. Chirtoca [CHGB 231630, 231631];
- Rîbnița district, Molochișul Mare commune, on limestone hills, 30.08.1934, T. Bilik [32291], 06.05.1987, L. Nikolaeva, teste T. Izverscaia [CHGB 224169], 10.08.1988, P. Pînzaru, tested by T. Izverscaia [CHGB 223340], 11.08.1995, P. Pînzaru [CHGB 233808], 14.06.1997, P. Pînzaru [CHUST-PP 4896]; Haraba commune, on limestone hills, 04.07.1987, K. Vitko, A. Railean [CHGB 217942, 217893],13.06.1997, P. Pînzaru [CHUST-PP 4895]; Sărăței village in Hărjău commune, on limestone hills, Nistru, 16.05.1984, K. Vitko [CHGB 213740].

The list of exsiccatae of *Gypsophila* × *moldavica* Pînzaru, nothosp. nova:

Republic of Moldova

- Chișinău municipality, Codru commune, in the author's private garden, 20.08.2019, P. Pînzaru [*Holotype*: CHGB 239989; *Isotype*: CHUST-PP 4890]; 20.08.2019, P. Pînzaru [CHUST-PP 4332, 4333], 24.07.2022, P. Pînzaru [CHGB 239991; IAGB 47742, 47743]; 13.09.2022, P. Pînzaru [CHUST-PP 4964]; 10.10.2022, P. Pînzaru [CHUST-PP 4947; CHGB 239990].

Results and discussions

As a result of floristic investigations on the vascular flora of the Republic of Moldova, a new hybrid species was described: *Gypsophila* × *moldavica* Pînzaru nothosp. nova (*G. glomerata* × *G. collina*).

The hybrid species *Gypsophila* × *moldavica* was formed as a result of cultivating together the species *G. glomerata* and *G. collina* in the author's private garden. The species *Gypsophila glomerata* Adams, until 1976, was indicated for South-Eastern Europe, Ukraine and the Caucasus [SĂVULESCU & RAYSS, 1926; PRODAN, 1953; BARKOUDAH & CHATER, 1964; GHEIDEMAN, 1975]. In 1976, in *Novosti Sistematiki Vyssih Rastenij*, the Russian botanist S. Ikonnikov mentioned 2 species: *G. glomerata* Adams, 1805 [= *G. globulosa* Boiss., 1867; *G. glomerata* var. *globulosa* (Steven) Schmalh., 1895], for the flora of the Caucasus region and *G. pallasii* Ikonn. sp. nova [= *G. glomerata* auct., non Adams: M. Bieb., 1808] for the flora of Crimea, without the morphological description of the species. Later, *Gypsophila pallasii* Ikonn. was accepted by other botanists [AKEROYD, 1993; ZIMAN, 1999; IKONNIKOV, 2004; CIOCÂRLAN, 2009; SÂRBU & al. 2013]. At the same time, the botanist

A. P. SEREGIN (2008), researching the flora of Crimea, recognized *G. glomerata* Adams (incl. *G. pallasii* Ikonn.). The species *G. glomerata* Adams had (previously) been recognized by T. GHEIDEMAN (1986) and A. NEGRU (2007), without indicating the presence of *G. pallasii* Ikonn. in the Republic of Moldova. Later, for the flora of the Republic of Moldova, only *Gypsophila pallasii* Ikonn. was cited [IKONNIKOV, 2004; IZVERSCAIA, 2016]. The difference between *G. glomerata* and *G. pallasi* was noticed while comparing the bracts, capitula and seeds: obovate-rounded, obtuse, eroded-toothed bracts, capitula of 8-15 mm in diameter and seeds with obtuse tubercles for *G. glomerata*, and for *G. pallasii* – oblong – rounded, entire bracts, capitula 3-12 cm in diameter and seeds with long conical, acute tubercles [IKONNIKOV, 2004; IZVERSCAIA, 2016]. According to S. IKONNIKOV (2004) – *G. pallasii* occurs on rocks and arid stony hills in R. Moldova (Bugeac), Crimea, the Caucasus (near Novorossiysk) and the Balkans.

In the habitats of Bugeac, in the Republic of Moldova, there are no rocks or stony hills, but there are areas with steppe vegetation, formed on clayey-sandy soils or on various chernozems. As a result of recent floristic research conducted in the field, as well as under *ex situ* conditions, it was found that *G. glomerata* grows in the Bugeac steppe, producing seeds with obtuse and not acute tubercles, and the bracts are broad-ovate, the lower ones are cuspidate at the tip, with finely eroded-denticulate margins, the diameter of the capitula on the same plant varies between 3 and 12 mm. In the summer of the current year, floristic research was carried out in Bulgaria (on the calcareous slopes on the Black Sea coast near the village of Topola, Kavarna municipality, Dobrich province) and in Romania (on the calcareous slopes around the Cotul Văii village in Albești commune, Constanța county and reserve Capul Doloșman in Jurilovca commune, Tulcea county, where *G. glomerata* plants with obtuse seed tubercles were found. As a result of the analysis of the aspect of the seeds of the species *G. glomerata*, *G. collina* and the hybrid species *G.* × *moldavica*, it was found that all these species produce seeds with obtuse tubercles, arranged in circles (Figure 8).

Gypsophila glomerata Adams, 1805, in Weber & Mohr, Beitr. Naturk. 1: 54. – Figure 1, 6.1, 7.1, 8a.

Syn.: incl. *Gypsophila pallasii* Ikonn. 1976, Novosti Sist. Vyssh. Rast. 13: 118, nom.nud.

The plants are 60-110 cm tall, usually with several stems, lignified at the base, branched at the top and with glandular hairs. The lower leaves are 3-veined, up to 10 cm long and 3-5 mm wide; in cultivated plants, the width of the lower leaves reaches up to 10 mm; the size of the other leaves gradually decreases towards the top of the stem. The inflorescences are globose capitula, 3-15 mm in diameter, with sessile flowers. Bracts – scarious, broad-ovate, the lower ones cuspidate, irregularly finely denticulate, with short glandular hairs. Calyx teeth with white membranous margin and short glandular hairs. Corolla 5 mm in diameter, white. Petals lanceolate, 4 mm long and 1 mm wide. Stamens exserted, with pink anthers. Fruit – capsule, unilocular, globose, dehiscent to the middle through 4 valves, with 2-4 seeds. Seeds dark brown, reniform-rounded, slightly compressed bilaterally, about 1.5 mm, with obtuse tubercles, arranged in circles.

Figure 1. *G. glomerata* – inflorescence, 05.08.2022

Biological and ecological peculiarities. Chamaephytic, xerophytic species. Blooms in July - October, the seeds ripen at the end of August - October. In the territory of the Republic of Moldova, it grows on clayey-sandy slopes, in steppe phytocoenoses; and in Bulgaria and Romania it grows on calcareous slopes.

Distribution in the Republic of Moldova (Figure 9). It occurs in the districts: Cahul (Giurgiulești, Cîșlița-Prut, Slobozia Mare, Văleni, Brînza and Colibaș communes), Vulcănești (Vulcănești, Etulia) and Taraclia (Musaitu, Ciumai).

General distribution. Greece, Bulgaria (Topola village in Kavarna municipality Dobrich prov.), Romania (Constanța county: Cotul Văii village in Albești commune and Tulcea county: nature reserve Capul Doloșman, Jurilovca commune), Ukraine (Odessa, Kherson, Mykolaiv, Donetsk oblast, Crimean peninsula), Russia (Stavropol krai), Caucasus (Novorossiysk), Turkey.

Gypsophila collina Ser. 1824, in Candolle, Prodr. 1: 352. – Figure 2, 6.2, 7.2, 8b

The plants are 60-70 cm tall, the stems – glabrous, lignified at the base. Leaves linear, one-veined, the lower ones up to 7 cm long and 3-5 mm wide, the others gradually smaller up to 2 cm long and 2 mm wide, with finely scabrous margins. Corymb inflorescences, flower stalks and peduncles glabrous. Bracts scarious, lanceolate, tapering towards the tip, with ciliated margins. Calyx teeth lanceolate, with white scarious and shortly ciliated margins. Corolla 5 mm in diameter. Petals obovate, 3 mm long and 1.5 mm wide, white, pale pink at apex. Stamens exserted, with pink-purple anthers. Fruit – capsule, globose, dehiscent to the middle through 4 valves, usually with 4 seeds, dark brown, reniform-rounded, about 1.5 mm, with obtuse tubercles, arranged in circles.

Figure 2. *G. collina* – inflorescence, 13.07.2018, Butuceni village, Orhei district

Biological and ecological peculiarities. Chamaephytic, xerophytic, calcicole species. Flowers in July - October, seeds ripen in September - October. In the territory of the Republic of Moldova, it grows in rock crevices and on friable limestone in the Dniester River Basin.

Distribution in the Republic of Moldova (Figure 9). It occurs in the districts: Florești (Ciutulești, Vărvăreuca communes and Stîrceni village, Cenușa village of Roșietici commune, Gvozdova village of Gura Camencii commune), Șoldănești (Rogojenii Vechi), Rezina (Țipova village of Lalova commune), Orhei (Butuceni and Morovaia villages of Trebujeni communes), Criuleni (Mașcăuți commune), Camenca (Camenca), Rîbnița (Beloci, Molochișul Mare, Haraba, Plopi, Sărăței), Dubăsari (Coicovo, Dubăsari, Doibani, Goian), Grigoriopol (Delacău).

General distribution: on rocks, stony slopes in Romania [CIOCÂRLAN, 2009; SÂRBU & al. 2013], Ukraine [ZIMAN, 1999].

Gypsophila* × *moldavica Pînzaru nothosp. nova (*G. glomerata* × *G. collina*) (Caryophyllaceae) – Figure 3, 4, 5, 6.3, 7.3, 8c.

The plants are perennial, glaucous. Stems 110-150 cm tall, lignified in the lower part, dichotomously branched from the base. Leaves opposite, sessile, linear, finely scabrous on the margins; the lower and middle ones 3-veined, 75-95 mm long and 5-8 mm wide; the upper ones one-veined, 25-30 mm long and 1.5-3 mm wide. Inflorescences – corymbiform dichasia. The middle and upper axes of inflorescences, bracts, floral peduncles and calyx are glandular-pubescent. Bracts broad-obovate, broad-lanceolate, cuspidate to tapering, whitish-scaly, on margins finely unevenly denticulate, with glandular hairs. Flower peduncles 2-5 mm long. Calyx 3-4 mm long, with 5 lanceolate teeth, ± as long as the tube, white membranous on margins. Corolla 5 mm in diameter, white. Petals lanceolate, 5 mm long and 1.3 mm wide.

Stamens grouped by 10, exserted, purple anthers. Styles 2, divergent, obviously longer than the stamens. Capsules shorter than the calyx, open by 4 valves, produce few fruits, with only one seed. Seeds dark brown, reniform-rounded, 1.5 mm, with obtuse tubercles.

Holotype: Republic of Moldova, Codru commune, Chișinău municipality, in the author's private garden, collected on 24.07.2022, P. Pînzaru [CHGB 239989]

Biological and ecological peculiarities. Hybrid species, chamaephyte, appeared under *ex situ* conditions, as a result of the hybridization of the species *G. glomerata* with *G. collina*. It blooms in July-October, seeds ripen in September-October.

Affinity. Differs from *G. glomerata* in the presence of 2-5 mm long pedicels (not sessile flowers), peltate inflorescence (not capitulum). Differs from *G. collina* in the lower and middle leaves with 3 veins (not one-veined), middle and upper axes of inflorescences with glandular hairs (not glabrous axes).

Distribution (Figure 9). The plants of the hybrid species appeared under *ex situ* conditions, as a result of the cultivation of the parental species in the author's private garden (Schinoasa Veche in Codru commune, Chisinau municipality).

Conclusions

The research carried out has confirmed the presence of the species *Gypsophila glomerata* Adams in South-East and East Europe, North Caucasus and Turkey.

The hybrid species *Gypsophila* × *moldavica* Pînzaru (*G. glomerata* × *G. collina*) possesses morphological characters of both parental species, rarely bears fruits, which produce only one seed, not 2-4; it is more vigorous and has higher decorative qualities.

The research was conducted with the support of NARD, within the project "Research and conservation of vascular flora and macromycobiota in the Republic of Moldova", 20.80009.7007.22.

Figure 3. *Gypsophila* × *moldavica* Pînzaru nothosp. nova, 05.08.2022.

Figure 4. *G.* × *moldavica* Pînzaru nothosp. nova – inflorescence, 05.08.2022.

Figure 5. Holotype of *Gypsophila* × *moldavica* Pînzaru (CHGB 239989).

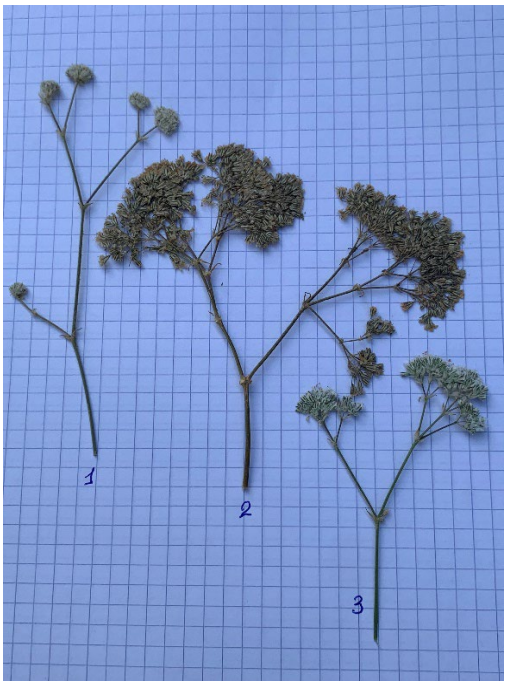

Figure 6. Inflorescence: 1. *G. glomerata*, 2. *G. collina*, 3. *G. × moldavica.*

Figure 7. Leaves: 1. *G. glomerata*, 2. *G. collina*, 3. *G. × moldavica.*

Figure 8. Seeds: a - *G. glomerata*, b – *G. collina*, c – *G. × moldavica.*

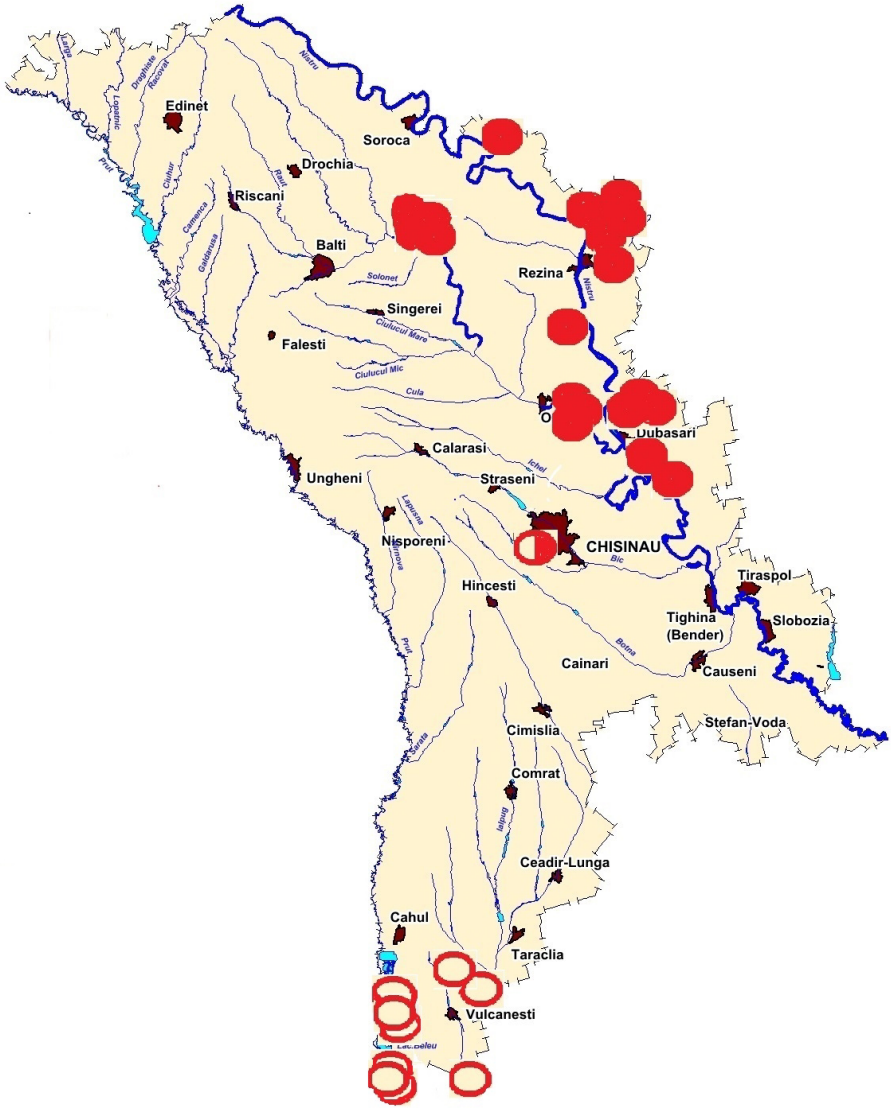

Figure 9. Distribution of the species *G. colina*, *G. glomerata, G.* × *moldavica* in Republic of Moldova.

Gypsophila collina Ser.

Gypsophila glomerata Adams

Gypsophila × *moldavica* Pînzaru

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The 75th Anniversary of the Biologist Georgeta Teodorescu

On March 7, 1947, botanist Georgeta Teodorescu (daughter of Gheorghe and Teodora Flenchea) was born in Roman, in Neamț County. In her hometown, she attended primary and secondary schools, and in 1964 she graduated with meritorious grades from the "Roman Vodă" high school. Between the years 1964-1969 she attended the courses of the Department of Biology-Botany (became the Faculty of Biology in 1990) within the Faculty of Biology-Geography. As a student, she stood out for her seriousness, passion and excellent results, so naturally she completed her studies with the best results in her cohort.

Immediately after graduating from the faculty, she held through competition a biologist position at the Botanical Garden in Iași, where she was intensively involved from the very beginning in the organizational activities of the institution recently (1962-1963) transferred to the site on the Copou hill. For a long period of time (1970-1996) she co-ordinated the activity in the World Flora section for which she initially elaborated and transferred to the field the scientific theme.

Starting from 1989 and until her retirement (2004) she coordinated the activity of the Greenhouse Complex, a sector with a remarkable taxonomic diversity that also involved a diversity of challenges. The work of inventorying and monitoring exotic plant collections is all the more valuable when we look at it in the context of that time, characterized by the lack of bibliography in the field and the difficult circulation of information. As the successor to the coordination of this sector I can appreciate firsthand the value of the work of inventorying and determining the species in the collections and I take this opportunity to express my gratitude for the dedication, tenacity and effort put in. Of the many contributions made to the development of the sector, we mention those related to the coordination and/or organization of various events. With her specific sensitivity and inclination towards the artistic field, she left her mark on the traditional exhibitions of "Azaleas and camellias" and the spaces intended for exotic greenhouse plants in the "Autumn Flowers" exhibitions. Landscaping, floral arrangements and all the decorative elements that highlighted the value of the exhibited plants continue to be valuable sources of inspiration.

Her charismatic presence, together with her oratorical and literary talent, made her valuable through the intense activity of popularizing information about the exotic plants grown in the Greenhouses. Over time, she conducted interesting interviews on radio and television shows and published numerous articles in the print media, appreciated by plant lovers.

The information and experience accumulated during the years of activity were kindly and professionally shared with colleagues from similar institutions (Botanical Gardens in Craiova, Jibou, Galaţi, Chişinău), where Mrs. Georgeta Teodorescu was requested as a scientific consultant.

Professional improvement and development was a permanent concern for biologist Georgeta Teodorescu. In 1998, she defended her doctoral thesis with the title "Research on the morphology and structure of some plants under parasitic conditions" (scientific supervisor - Professor Constantin Toma, PhD in Biology, member of the Romanian Academy).

As valuable professional she permanently collaborated with her colleagues from the Department of Botany of the Faculty of Biology, where she worked as an associate teacher in the discipline Morphology and plant anatomy, during the period 1994-1996. However, she was involved for a much longer period of time in the activity of educating students, by coordinating or guiding the elaboration of bachelor's or master's theses in fields of study related to the specifics of the Botanical Garden.

In the 35 years of activity, she has published as the sole author or in collaboration, in specialized magazines from the country and abroad, about 80 scientific works in different fields of study: morphology and anatomy, blastogeny, chorology, dendrology, phenology, floriculture. The articles focused on groups of exotic plants (palm trees, ferns, azaleas and camellias, useful species) and the small volume "Crizantema" (in collaboration with Profira Vidrașcu) remain very useful as landmarks in the scientific activity in Botanical Garden of Iași. She participated in numerous symposia where she gave oral communications or appreciated conferences. She was also a collaborator in 6 research projects in which she was involved with tenacity and professionalism.

She was a member of the editorial board of volumes 2 (1982) and 3 (1987) of "Collection of Studies and Articles of Biology" (now Journal of Plant Development) and "Ghidul Grădinii Botanice" from Iași (second edition, revised, 1988) .

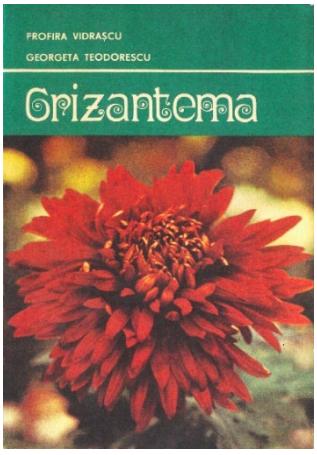
Biologist Georgeta Teodorescu's love for plants found its best expression in the volume "Vegetable extravagances", published in 2004, valuable for the wealth of information, the attractive style of presentation and the unique selection of illustrations.

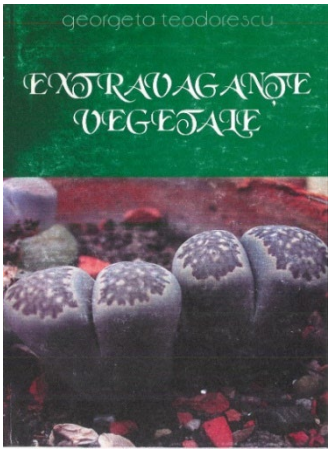
For all her collaborators, Mrs. G. Teodorescu remains a model of passion, sensitivity, artistic spirit and tenacity, qualities that helped her to meet the challenges determined by the status of a biologist in a Botanical Garden. Even after her retirement, she remained close to the institution where she worked with devotion and continues to collaborate with younger colleagues who are still active.

On my personal behalf, as well as that of the Botanical Garden Iași staff, on the occasion of her 75th anniversary, we wish her good health, beautiful moments with her beloved ones, and energy to complete the projects she works on with great dedication.

Happy anniversary!

Camelia IFRIM
"Alexandru Ioan Cuza" University of Iaşi, "Anastasie Fătu" Botanic Garden





BOTANIST DR. ION SÂRBU (17.09.1933-26.07.2022)

Dr. Ion Sârbu was one of those scientists who, throughout his entire professional career, was concerned with the study of life sciences, especially the world of plants, a field in which he obtained remarkable, indisputable, extensive and of great depth results.

Born at the beginning of autumn of the year of 1933, in Suceveni village, Tudor Vladimirescu commune, the county of Galaţi, he attended the primary school in his native village, the secondary school and College/Normal School of Teachers "Costache Negri" in Galaţi, in order to complete his higher education in Iași, at the Faculty of Natural Sciences-Geography, Biology-Geography section, of the "Alexandru Ioan Cuza" University, which he graduated in 1963. He took his state exam in February 1964, being declared "Diplomat University", mainly in Biology, but secondary in Geography.

As a result, he became a professor of biology and geography in his native commune; between 1964 and 1967 he was also a specialist inspector in Biology and Geography, in the Bujor district, Galaţi Region.

In 1967, at the insistence of his Professor Constantin Burduja, Mr. Ion Sârbu transferred with his job (and moved with his family) to Iaşi, working as a biologist at the Herbarium of the Botany Department, Faculty of Biology. Later on, in 1975, he was transferred to the Botanical Garden "Anastasie Fătu" in Iași, where he would work until his retirement in 2003.

Here, he laid the foundations and coordinated the works of the "Flora and Vegetation of Romania" section, took care of the institution's herbarium and coordinated the scientific periodicals "Collection of Studies and Articles of Biology" / "Bulletin of the Iași Botanical Gardens", "Catalog of Seeds and Spores", but also the aperiodic publication "Flora Exsiccata Moldaviae et Dobrogeae".

Starting from 1973, he studied his native region from a botanical point of view, in order to defend his doctoral thesis in 1978, *Flora and Vegetation of the Chinejii and Prut Basins between Rogojeni-Mastacani*. Thus, he gained the title of PhD in Biology at the University "Alexandru Ioan Cuza" Iași, in 1978, under the direction of the same Professor and mentor, Constantin Burduja.

Professionally, Ion Sârbu follow a path that was not exactly easy, but which with a lot of work, perseverance, giving up and sacrifices in his personal life, highlighted his tenacity and native intelligence. Thus, he followed an upward path, full of professional achievements, from biologist to scientific researcher, the 1st degree, with his first studies on the taxonomy and chorology of *Galanthus* spp. or *Fagus* spp. in Romania, to the last ones, such as some critical analysis and synthesis works on the vascular flora, natural vegetation, terrestrial or aquatic habitats of Romania.

Active spirit, devoted to his profession, being endowed with special physical and intellectual abilities, a fine and attentive observer of life in nature, he materialized his own observations by publishing more than 120 scientific works and 22 books and book chapters, in

Romania or abroad, which will preserve his memory in posterity as one of the most prominent personalities of contemporary botany in Romania and beyond.

Throughout his scientific activity as a botanist, Dr. Ion Sârbu was involved as a collaborator in 38 national or international projects, thus publishing 128 scientific works and 22 books and chapters of books, alone or in collaboration with Romanian or foreign botanists.

The significant contributions of Dr. Ion Sârbu in approaching the taxonomic research, both at national and international level, are supported by the collaboration of some fundamental works, such as: *Vascular Plants of Romania*. *Illustrated Field Identification Book* (Bucharest, 2013), *The Map and The Red List of the Danube Delta Biosphere Reserve* (Tulcea, 1994 and 2000), *Les Associations Végétales de Roumanie*. *Les Associations Anthropogènes* (Cluj-Napoca, 2012), *Atlas Florae Europaeae* (Helsinki, 1990-2018), and the electronic *Data Base of the Flora Europaea* (1994-2021), and many other botanical operas.

As recognition of his important scientific activity, Mr. Ion Sârbu was granted with "Emanoil Teodorescu" Prize of the Romanian Academy, in 2006.

He activated also as a member of some professional associations in Romania, such as the Romanian Phytosociology Society, the "Anastasie Fătu" Dendro-Ornamental Association, or the Romanian Society of Biology.

Sensitive soul, communicative, friendly, open to dialogue, reliable, always ready to help anyone in difficult moments, he was fully appreciated with respect and considerations by his collaborators, both for his professional qualities and for his spirit of humanity, fairness and understanding.

It is obvious that I cannot express in sufficiently, comprehensive words, how much meant Mr. Ion Sârbu, both for me personally and for us, the younger generation of Romanian botanists. By his calm and warm way of speaking, by the kindness and competence with which he supported us every time we asked him for advices, books, botanical references, scientific works, maps or unraveling the secrets of any mountain or forest in our country, and many others, he will remain a great, dear and good MAN, for most of us.

By his achievements, Mr. Ion Sârbu was and will be a model of human, moral and intellectual conduct, a worthy model to follow for us, aspiring botanists, not only from Iaşi, but even from Romania.

For everything he did for Romanian botany, as well as for the support given to each of us, I thank him from the bottom of my heart, both personally and on behalf of my colleagues from the Botanical Garden "Anastasie Fătu", University "Alexandru Ioan Cuza" of Iaşi.

Personally, I met Mr. Ion Sârbu on an early autumn day in the somewhat distant year of 1990. Then he took me with him on my first field trip as a new employee of the Botanical Garden, a trip to the Danube Delta in Romania, collecting numerous plant specimens and seed samples. This is how I met the man who was to become a true mentor and model of scientific professionalism in my later work as a botanist. In the years that followed, we went many field trips, where, in addition to the originally proposed goals, we made countless plant identifications, had many botanical discussions or he showed me some of the protected natural areas or important points of botanical interests of Romania.

My colleagues and I have lost not only a special colleague, a close collaborator and an imposing researcher, but also a honorable friend, a man full of humanity and compassion for the troubles of others, a man around whom we all always felt relaxed and eager to ask him more and more about our botanical concerns.

The grieving family, his wife, children and grandchildren, have lost a good and devoted husband, a family man and a wise grandfather. He loved them all very much, surrounding them with parental love and care (sometimes he would tell us about the vacations he took with his

family, into the Danube Delta or on the coast of the Black Sea, lodging everyone in tents, with all the nostalgic memories that remain in such moments; or he would tell us other episodes from the lives of his loved ones, which he told us in the tone of wise father or grandfather …). The disappearance of him from his family leaves a void that is hard to replace as moral support permanently given to those close to him.

Let us pray to God to rest him in peace, and let us keep him in our memory until the day when we follow the same path.

Adrian OPREA
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iaşi

JOURNAL OF PLANT DEVELOPMENT
GUIDE TO AUTHORS

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Journal of Plant Development is the official scientific journal of the "Anastasie Fătu" Botanical Garden, which belongs to "Alexandru Ioan Cuza" University of IAŞI, ROMANIA. It was first published in 1979 (at that time as "Culegere de Studii şi Articole de Biologie"). The new series begins in 1993 under the name "Buletinul Grădinii Botanice Iaşi". From 2008 on, it has been published under its present name "**Journal of Plant Development**". It appears in one volume, with one or two issues per year.

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The papers will be published only in a foreign language (English), structured as follows: title, authors, affiliation of the authors, abstract, keywords, introduction, material and method, results & discussions, conclusions, acknowledgements, references, tables, figure captions.

Title should be a brief phrase describing the contents of the paper.

Authors names would not be abbreviated, capitals for surname (family name) and no capitals for first name (except initial letter). Each author name would be accompanied by a complete address, as a footnote on the first page. The affiliation should be provided in the following order: university (institution) name; faculty/department name; number and street name; city; country and email address. One of the authors should be designated as the corresponding author.

Abstract should be concise informative and completely self-explanatory, briefly present the topic, state the purpose of the research, indicate significant data, and point out major findings and conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list. Non-standard or uncommon abbreviations should be avoided but, if essential, they should be defined at their first mention in the abstract itself.

Key Words: five to seven words, the most important ones, in alphabetical order, after someone could discover your paper on the internet engines. Key words should not repeat the title of the manuscript.

The **Introduction** should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines. The introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Methods in general use need not be described in detail.

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Conclusions should spell out the major findings of the work and may include some explanation of the significance of these conclusions and what further research should be done. Authors should include a general interpretation of the results in the context of current evidence, and not restricted to that which supports the findings of the present study.

Notes on contributors in maximum 65 words, provide short biographical notes on all contributors.

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	□	**Chapters in books:** author(s), year, title, pages, a point sign, followed by "In": author (s) of the book, city, publishing house, number of pages. TUTIN T. G. 1996. *Helleborus* L. pp. 249-251. In: TUTIN T. G. & al. (eds). *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, XLVI, 581 pp.
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