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Root colonization by micromycetes in ten *Asteraceae* species from Cluj County

Crişan Ioana[1], Vidican Roxana[1*], Stoie A.[1], Stoian V.[1]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania

*Corresponding author. Email: roxana.vidican@usamvcluj.ro

Abstract Symbiotic root fungal biotrophs are cryptic plant colonizers that can bring several nutritional-related benefits to their hosts. Aim of this study was to assess the level of colonization by common micromycetes such as: arbuscular mycorrhizae (AM), fine root endophytes (FRE) and dark septate endophytes (DSE) across a plant genotype gradient represented by ten *Asteraceae* species from Agro-Botanical Garden UASVM Cluj-Napoca. Evaluation was conducted on stained roots using optical microscopy. Results showed the highest AM colonization intensity in *Liatris spicata* and highest arbuscularity in *Ageratum houstonianum*. Highest DSE frequency was found in *Aster dumosus*. Fine root endophytes were observed only in three annual species and were particularly abundant in roots of *Tagetes erecta*. This is the first sampling for comparative assessment of some mutualistic micromycetes in *Asteraceae* species from local climate. Results obtained completed by data from literature suggests that pattern of interspecific range of variability for fungal endophytic colonization might have underlaying climatic significance.

Key words

symbiosis, hyphae, genotype gradient, aestival landscape

From evolutionary perspective, *Asteraceae* family is relatively young, but currently represents the largest family of flowering plants with around 25000 species distributed worldwide. It includes many economically important food crops, herbal and medicinal species as well as ornamentals [8]. It is one of the best-represented plant families in urban ecosystems since are extensively used in landscaping while many of them are good melliferous plants providing rewards for insects from late spring to late autumn [20]. In Romania, *Asteraceae* are emblematic garden ornamentals for summer season.

Several groups of micromycetes that colonize plant roots are known for being able to bring nutritional benefits for their hosts. In this sense the evidence particularly for arbuscular mycorrhiza fungi (AMF) ability to enhance phosphorus uptake of plants is extensive [3]. In addition, dark septate endophytes (DSE) were shown to also act as plant growth promoters in presence of organic nitrogen [17]. Fine root endophytes (FRE) have to be further examined to find more about their role in relation with plant growth but some evidence indicates they might also enhance nutrient uptake [10].

Each of these main micromycetes groups can be examined and recognized under microscope based on distinct morphologic features (Fig. 5).

Arbuscular mycorrhizae (AM) is an association of plant roots with obligate biotrophic fungi from phylum *Glomeromycota*. These colonize root cortex spreading in intercellular and intracellular space with aseptate hyphae much thicker than FRE. Inside cells the fungi are forming short-lived modified haustoria called arbuscules which invaginate plasmalemma providing surface area for nutrient exchange between symbionts. Spores form inside and outside roots. There are several other structures that make them rather distinctive from different groups of root fungal colonizers [18].

Fine root endophytes (FRE) are a group of micromycetes that also form close intraradical associations called “mycorrhizas” with suitable vascular plants but also probably forming similar endophytic mutualistic associations – “paramycorrhizal” in thalli of some liverworts, hornworts and rhizoids of bryophytes. Although FRE are classified in genus *Planticonsortium* from subphylum *Mucoromycotina*, they also form arbuscules inside intra-cellular space of middle and inner cortex of the root, but present thinner “trunk” compared to AM. FRE present very fine hyphae with small swellings along their length. In addition, they form feather-like, fan-like and palmate structures. Spores are very small. Their diversity remains in most part unknown [10, 11, 19] which makes them interesting for future research.

Despite some similarities with AM, the FRE appear to be more closely related to *Endogonales* from subphylum *Mucoromycotina* - known for including

fungal species able to participate in either ecto- or endomycorrhizal associations with diverse vascular and non-vascular plants, than with *Glomeromycota* which is exclusively endomycorrhizal with a large number of plants both non-vascular (liverworts and hornworts, except mosses) as well as tracheophytes (ferns, gymnosperms, angiosperms) [5, 7, 10, 11, 12].

Dark septate endophytes (DSE) are a group of diverse facultative biotrophic *Ascomycetes* that is not monophyletic. They are characterized by sterile, dark septate hyphae developing inside heathy plant roots. DSE can present variable dark pigmentation and form microsclerotia inside plant cells. They colonize intra and intercellular space in roots of vascular plants without causing known pathologies instead enhancing plant performance [9, 17].

Aim of the study was to assess the functional assembly and identify the range or variability in local climate for co-occurring root symbiotic micromycetes along a plant genotype gradient represented by different *Asteraceae* species, from Agro-Botanical Garden UASVM Cluj-Napoca, using optical microscopy.

Material and Methods

Biologic material for this study was obtained from Agro-Botanical Garden UASVM Cluj-Napoca Romania. The garden is situated in temperate continental climate at an altitude of 380-430 m, experiencing an average annual temperature 8.1°C and average annual rainfall of 635 mm [21]. Soil type is clay loam with good NPK supply.

On 7th August 2018, were collected roots from ten *Asteraceae* species with ornamental value, at anthesis: *Ageratum houstonianum* Mill., *Aster dumosus* L., *Coreopsis tinctoria* Nutt., *Cosmos bipinnatus* Cav., *Helianthus annuus* L., *Liatris spicata* (L.) Willd, *Rudbeckia hirta* L., *Tagetes erecta* L., *Tanacetum vulgare* L., *Zinnia elegans* Jacq. Nine of these species are native to North America with the exception of *Tanacetum vulgare* which is native to Eurasia [23].

Roots were prepared for microscopic examination using an adaptation of two methods [15, 16]. The method described here was modified in order to be suitable for *Asteraceae* roots, which are not easily flatten after mounting for observation if the segments haven't been softened enough. This can be achieved either by longer clearing treatment or speeding up the process by heating.

Immediately after collecting, samples received a code to ensure objectivity in analysis. Then, roots were washed with tap water using a tea sieve and placed in mini-jars with 15% NaOH solution for 3 days, then transferred to a solution of 5% KOH for another 2 days. Roots were not heated but left for slow clearing. It was necessary a longer time to achieve a satisfactory softening of the roots. Then, roots were gently washed with vinegar-water solution and finally transferred in a staining solution of 5% blue ink + 5% vinegar (9°) (Fig. 1).

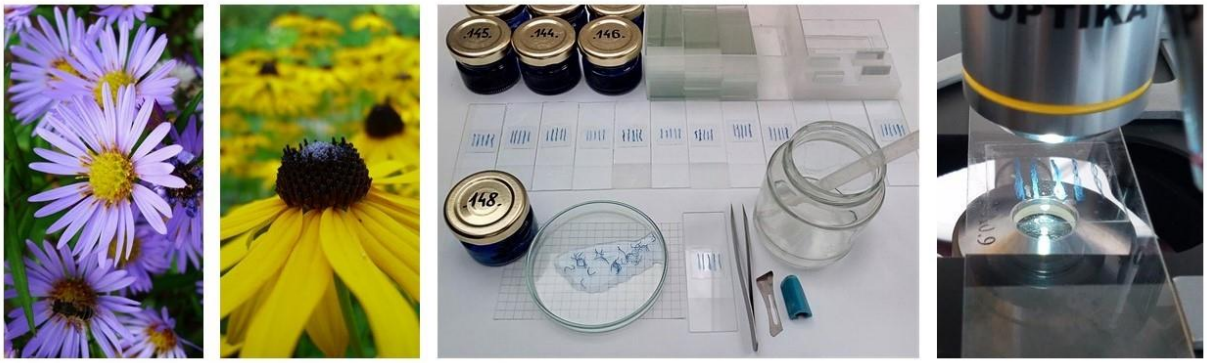


Fig. 1. *Asteraceae* flowers in Agro-Botanical Garden UASVM Cluj-Napoca
and protocol for assessing root fungal endophytes under optical microscope (Original)

Root samples were prepared for microscopic examination using squash technique and observed using Optika microscope at 100×-400×.

A number of 200 random root segments of 1 cm were assessed for presence of: arbuscular mycorrhizae *(Glomeromycota)* abbreviated AM, fine root endophytes *(Mucoromycota)* abbreviated FRE and dark septate endophytes *(Ascomycota)* abbreviated DSE.

Arbuscular mycorrhizae colonization was evaluated according to Trouvelot method [14] while indicators for arbuscular mycorrhizae were calculated using Mycocalc software [22]. AM vesicles and intraradical spores were considered as advanced stage colonization structures, and their frequency taken together, because distinguishing between them can be difficult in some cases and might be prone to error.

For the other two categories of root endophytes, frequency was also calculated as

percentage of root segments presenting colonization out of total number of segments analyzed per species.

Parameters subject to analysis were:

- F% = frequency of occurrence for AM hyphae, AM spores + vesicles, DSE, FRE,
- m% = intensity of the mycorrhizal colonization in the root fragments,
- M% = intensity of the mycorrhizal colonization in the root system,
- a% = arbuscule abundance in mycorrhizal parts of root fragments,
- A% = arbuscule abundance in the root system.
- range of variability (R = max – min) for each parameter mentioned above.

Results and Discussion

From table 1 can be observed that plant species which presented the highest frequency of AM colonization were *Liatris spicata* with all segments analyzed showing presence of *Glomeromycota* fungi, followed by *Rudbeckia hirta* and *Aster dumosus.* Lower colonization frequency was identified in *Coreopsis tinctoria* and *Tagetes erecta*. Intermediate frequency of occurrence compared to these lower and upper limits was identified in several therophytes named in descending order: *Ageratum houstonianum, Zinnia elegans, Cosmos bipinnatus* and *Helianthus annuus.* Within same interval for frequency of AM occurrence is situated also *Tanacetum vulgare.*

Table 1

Root colonization of ten *Asteraceae* species from Cluj county, Romania (August 2018)

Species	Coarse AM colonization frequency (F%)	AM vesicles and spores frequency (%)	FRE Frequency (%)	DSE Frequency (%)
Ageratum houstonianum Mill.	85	30	-	20
Aster dumosus L.	90	30	-	60
Coreopsis tinctoria Nutt.	65	5	35	20
Cosmos bipinnatus Cav.	80	10	-	10
Helianthus annuus L.	80	10	-	5
Liatris spicata (L.) Willd	100	75	-	5
Rudbeckia hirta L.	95	55	-	20
Tagetes erecta L.	35	30	55	55
Tanacetum vulgare L.	80	30	-	5
Zinnia elegans Jacq.	85	35	5	10

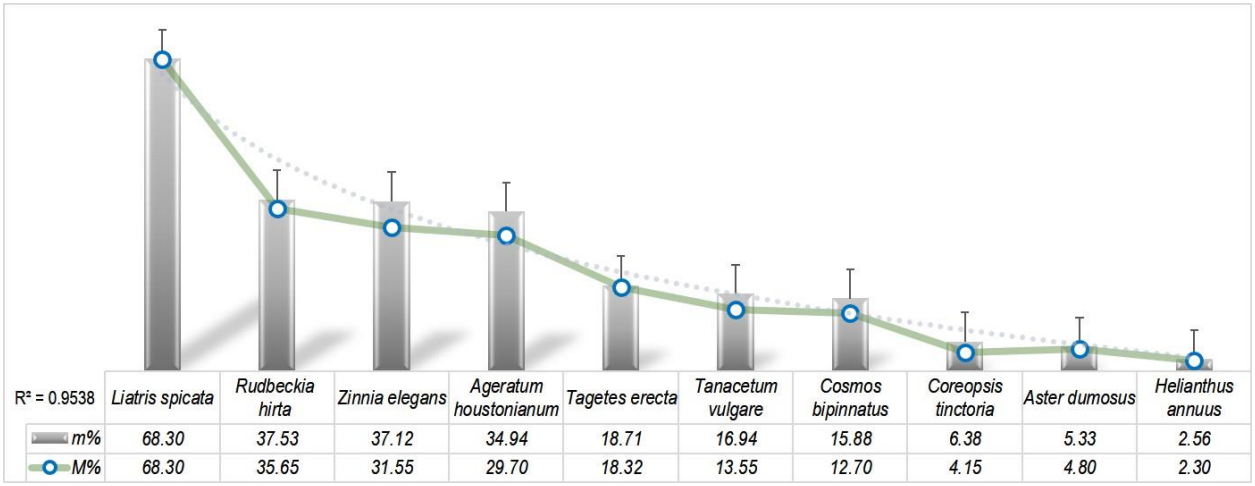
AM – coarse arbuscular mycorrhizae, FRE – fine root endophytes, DSE – dark septate endophytes

Higher frequency of occurrence for advanced colonization structures produced by arbuscular mycorrhizal fungi such as vesicles and intra-radicular spores, was associated with higher AM colonization frequency for two species: *Liatris spicata* and *Rudbeckia hirta*. This was not the case for *Aster dumosus* in which case frequency of AM vesicles and spores was not associated with higher frequency of AM colonization. Lowest frequency of AM intra-radicular spores and vesicles was recorded for *Coreopsis tinctoria.* Interestingly, *Tagetes erecta* which had the lowest frequency of AM colonization did not show the lowest frequency of AM vesicles and spores.

FRE were observed only in root segments of three annual species, with highest occurrence in *Tagetes erecta* followed by *Coreopsis tinctoria* and *Zinnia elegans.* As it can be seen in table 1, in *Tagetes erecta* FRE occurs simultaneous with lower AM levels perhaps because both provide similar services to plant. The same could be for *Coreopsis tinctoria.*

DSE were observed in roots of all species analyzed. Highest DSE frequency was obtained for *Aster dumosus* followed by *Tagetes erecta*. Lowest DSE frequency was found in two perennials: *Liatris spicata, Tanacetum vulgare* and in one annual, namely: *Helianthus annuus.* The rest of the species analyzed had intermediate frequency of DSE colonization, situated between 10-20%.

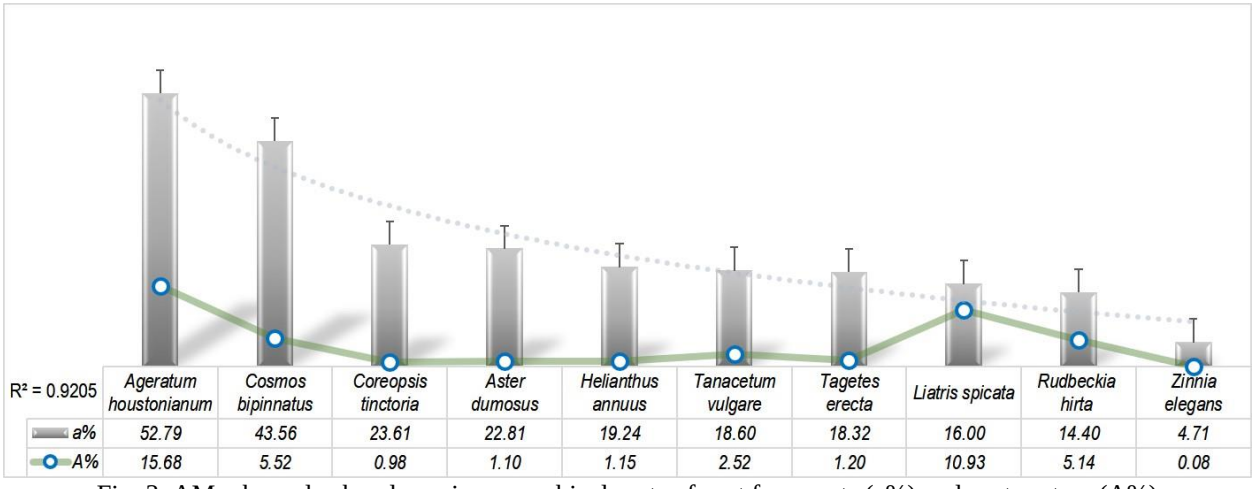
Highest intensity of colonization in root fragments and root system was found in *Liatris spicata* followed by hemicryptophyte plant *Rudbeckia hirta*. Lowest intensity was identified in *Aster dumosus* and *Helianthus annuus*. Similar values, between intensity of arbuscular mycorrhizae in root fragments and root system indicates to a uniformly development and spreading of mycorrhiza (particularly hyphae of arbuscular mycorrhiza fungi) in rhizosphere and inside root cortex. As the differences between the two indicators of intensity increases it becomes evident a less homogenous presence of the fungus in the root system, with only spares areas of colonization along the roots or intense patches of colonization alongside completely mycorrhizal-free root areas. Although strikingly different in value, steady spreading of hyphae inside roots indicated by relatively equal m%≈M%, was found in *Liatris spicata, Tagetes erecta* and *Helianthus annuus* (Fig. 2).



R^2 = 0.9538	*Liatris spicata*	*Rudbeckia hirta*	*Zinnia elegans*	*Ageratum houstonianum*	*Tagetes erecta*	*Tanacetum vulgare*	*Cosmos bipinnatus*	*Coreopsis tinctoria*	*Aster dumosus*	*Helianthus annuus*
m%	68.30	37.53	37.12	34.94	18.71	16.94	15.88	6.38	5.33	2.56
M%	68.30	35.65	31.55	29.70	18.32	13.55	12.70	4.15	4.80	2.30

Fig. 2. Intensity of arbuscular mycorrhizae in root fragments (m%) and root system (M%) along a genotype gradient comprised by ten *Asteraceae* species from Cluj county, Romania (2018)

When it comes to arbuscularity, it was noted that it reaches highest value in *Ageratum houstonionum*, followed by *Cosmos bipinnatus*, and lowest in *Zinnia elegans*. The large difference between abundance of these exchange units in root fragments and root system identified as a% vs. A% in figure 3, can be taken as an indicator of exchange rate between plants and arbuscular mycorrhizal fungi. In some cases, only a few root fragments presented arbuscules, but they nevertheless sometimes were very abundant, while in other cases a few arbuscules were scattered or disposed sparsely across a large number of analyzed root segments indicating to difference perhaps related to plant needs and metabolic rate.



R^2 = 0.9205	*Ageratum houstonianum*	*Cosmos bipinnatus*	*Coreopsis tinctoria*	*Aster dumosus*	*Helianthus annuus*	*Tanacetum vulgare*	*Tagetes erecta*	*Liatris spicata*	*Rudbeckia hirta*	*Zinnia elegans*
a%	52.79	43.56	23.61	22.81	19.24	18.60	18.32	16.00	14.40	4.71
A%	15.68	5.52	0.98	1.10	1.15	2.52	1.20	10.93	5.14	0.08

Fig. 3. AM arbuscule abundance in mycorrhizal parts of root fragments (a%) and root system (A%) along a genotype gradient comprised by ten *Asteraceae* species from Cluj county, Romania (2018)

Analyzing the overall variability range for colonization parameters by micromycetes, several interesting facts can be noticed (Fig. 4). Firstly, variability range for AM spore and vesicle frequency (R AM sv F% = 70) is higher than variability range for colonization frequency (R AM F% = 65). However, the range of AM frequency maintains rather similar with DSE frequency range (R DSE F% = 55) across the ten *Asteraceae* species studied. Although different in value, the variability range for arbuscularity in root system maintains at the lowest level compared to all the parameters studied: R A% = 15.6, while the variability range for arbuscule abundance in root fragments has higher levels R a% = 48.8. Similar variability range can be noticed for AM intensity of colonization in root fragments (R m% = 65.74) and root system (R M% = 66).

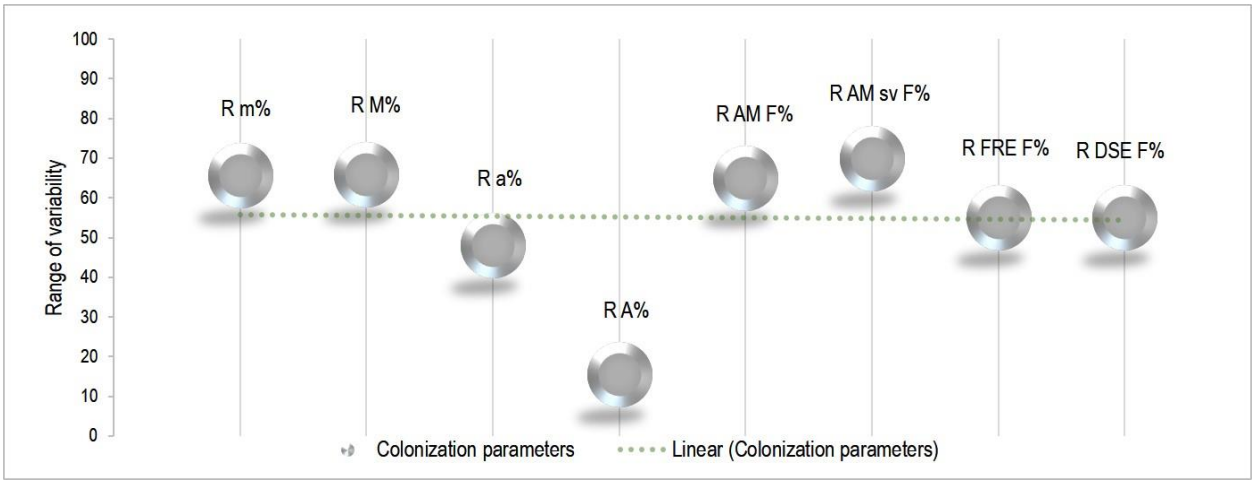


Fig. 4. Range of variability for colonization parameters across ten *Asteraceae* species from Cluj county

Some images with stained roots from *Asteraceae* species studied can be seen in figure 5, together with identity attributed in each case.

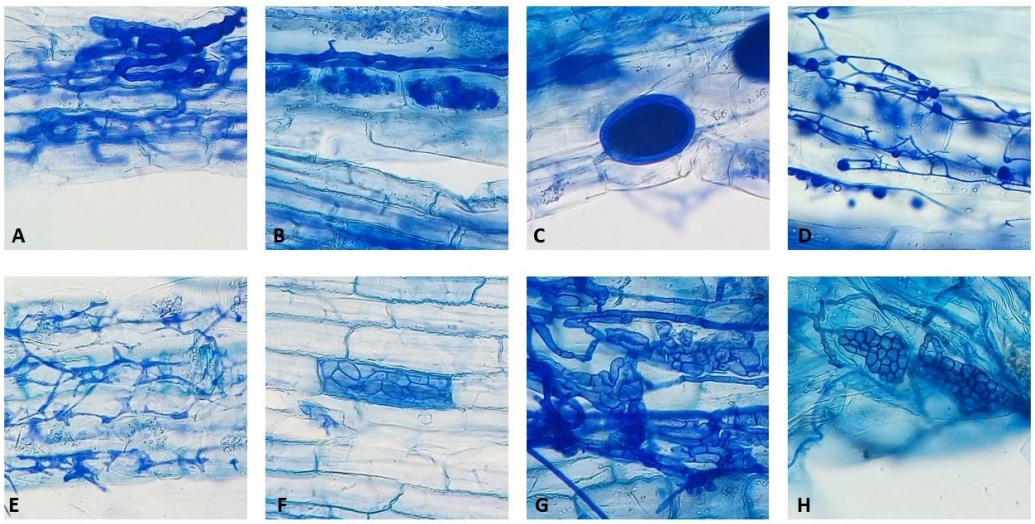


Fig. 5. Micromycetes inside stained roots of some *Asteraceae* species, identified as: a) AMF entry point in *Ageratum houstonianum*; b) AM arbuscules in *Liatris spicata*; c) AM spores and vesicles in *Helianthus annuus*; d) FRE in *Tagetes erecta*; e) FRE in *Coreopsis tinctoria*; c) DSE microsclerotia in *Tagetes erecta*; g) DSE colonizing root of *Tagetes erecta*; h) DSE in *Rudbeckia hirta* (Original)

Previous studies on *Asteraceae* species from contrasting climates: tropical, subtropical, arid, temperate, arctic, showed that level of root colonization by fungal endophytes always presents some interspecific variability although of different range in each case.

A study conducted in same temperate pedo-climatic conditions of Romania, for two geophyte *Asteraceae*: *Dahlia variabilis* and *Helianthus tuberosus* no FRE presence was detected in roots when plants researched anthesis, while DSE was found in both species. Similar as was observed for *Liatris spicata* from current study, higher frequency of AM colonization in *Dahlia variabilis* was also associated with higher vesicle and intra-radicular spore frequency [4]. In a study conducted in Floristic Reserve from Cuba, situated in tropical climate, in regards with five *Asteraceae* taxa from genera *Aster*, *Erigeron*, *Pectis* and *Sachsia* was found DSE presence in all species, while AM showed high colonization rates particularly in dry season [13]. Nine *Asteraceae* species from Yungas subtropical ecosystem of Argentina, a region with high floristic diversity, were assessed in relation with percentage of AM colonization. Results indicated <50% colonization in species from genera *Taraxacum*, *Jungia*, *Stevia*, *Gnaphalium*, and >50% in species from genera *Cirsium*, *Siegesbeckia*, *Bidens* and *Tagetes* [2]. Similar with the current study was noticed a wide variation of natural root colonization by AM across *Asteraceae* species studied. By contrast, in case of six *Asteraceae* species from semi-arid and arid climate of Saudi Arabia - different locations, although was

registered some variation in AM root colonization across species, it was not as wide. According to the cited research, highest colonization was found in *Conyza bonariensis* (65%) while the lowest in *Asteriscus graveolens* (22%) and *Vernonia schimperi* (22%). There were also identified some differences in root colonization for same plant species linked to distinctive soil properties from locations of study [6]. In eight *Asteraceae* species belonging to genera: *Arnica*, *Erigeron* and *Taraxacum* from arctic climate region of Canada, percentage of AM colonization of root length was >60%, and in addition the plants presented colonization by FRE exhibiting about three distinctive morphologies [1].

Thus, based on the results obtained and literature cited above highest range of variability for fungal endophytic colonization for *Asteraceae* species was reported for subtropical climate, followed by temperate climate. Within the middle of this range was data reported for hot arid environment and arctic climate. Lowest range of variability in root colonization level across species was identified in tropical climate during dry season. Thus, although level of root colonization by fungal endophytes always presents variability among species, it might have climatic-linked significance.

Conclusion

In this comparative study conducted on ten annual and perennial ornamental *Asteraceae* species was shown that highest AM colonization intensity occurred in *Liatris spicata* and highest abuscularity in *Ageratum houstonionum*. Highest DSE frequency was found in *Aster dumosus*. Fine root endophytes were observed only in root segments of three annual species and were particularly abundant in roots of *Tagetes erecta*.

Lowest range of variability for colonization parameters across the ten *Asteraceae* species was identified for AM arbuscule abundance in root system. Similar range of variability across species resulted for three parameters of AM colonization: frequency of colonization, intensity of colonization in root fragments and root system.

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