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Seasonal dynamics of fungal colonisation of *Plantago altissima* roots in a water-fluctuating wetland

Sezonska dinamika glivne kolonizacije korenin visokega trpotca v presihajočem mokrišču

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Abstract: Fungal colonisation in an amphibious plant species from an intermittent lake may differ significantly among plant specimens growing under contrasting water regimes. We examined the effect of the presence of surface water on the level of fungal colonisation in tall plantain (*Plantago altissima* L.). We investigated the presence and abundance of arbuscular-mycorrhizal (AM) structures, dark-septate-endophyte (DSE) fungal structures, and plant growth parameters. The observed overall frequency of fungal structures during the vegetative season was relatively high, ranging from 50% to 90%. Mycorrhizal frequency was little affected by water level fluctuations. However, it dropped significantly at the end of the season in the senescence phase. The densities of arbuscules, vesicles, and hyphal coils reached the highest levels in peak season in June and July. The density of DSE structures, namely microsclerotia, showed similar dynamics over the season as for AM structures. Mycorrhizal frequency was positively related to leaf water content.

Keywords: intermittent lake, *Plantago altissima*, roots, fungal colonisation, arbuscular mycorrhiza, dark septate endophytes

Izvleček: Glivna kolonizacija amfibijskih rastlinskih vrst na presihajočem jezeru se lahko znatno razlikuje med rastlinami, ki rastejo v razmerah različnega vodostaja. Preučili smo učinek prisotnosti površinske vode na raven glivne kolonizacije pri visokem trpotcu (*Plantago altissima* L.). Raziskali smo prisotnost in številčnost struktur arbuskularne mikorize (AM), struktur temnih septiranih endofitov (DSE) in parametre rasti rastlin. Pogostnost glivnih struktur med je bila med rastno sezono rarmeroma visoka, saj je segala od 50 % do 90 %. Pogostnost mikorize je ostala na isti ravni ne glede na spremembe vodostaja, vendar se je ob koncu sezone v fazi senesence znatno znižala. Gostota arbuskulov, veziklov in svitkov je dosegla najvišjo raven v juniju in juliju. DSE strukture, mikrosklerociji, so imele podobno dinamiko kot AM strukture. Pogostnost mikorize je bila pozitivno povezana z vsebnostjo vode v listih.

Ključne besede: presihajoče jezero, *Plantago altissima*, korenine, glivna kolonizacija, arbuskularna mikoriza, temni septirani endofiti

Introduction

Water regime shapes physical, chemical, and biological processes in wetlands (Cronk and Fennesy 2001). Intermittent wetlands are characterised by extreme water level fluctuations that affect habitat conditions in time and space (Dolinar et al. 2016). Alternations of flooded and dry periods result in a unique flora with specific adaptations that enable plants to cope with these outstanding conditions (Braendle and Crawford 1999, Gaberščik et al. 2003, Urbanc-Berčič et al. 2005, Šraj et al. 2006). Roots of the majority of wetland species are colonised by fungi (Escudero and Mendoza 2005, Stevens et al., 2010). Variations in mycorrhizal colonisation among different species in wetlands are crucial for structuring fen plant communities (Cornwell et al. 2001). Surveys of plant root systems of these species revealed the presence of dark septate endophytes (DSE) and arbuscular mycorrhizal fungi (AM) (Šraj et al. 2006, Gaberščik et al. 2017). DSE are common especially in habitats with frequent stress conditions (Mandyam and Jumpponen 2008). DSE are a diverse group of Ascomycetes that colonises root tissues inter- and intracellularly and form clusters of inflated, rounded, and thick-walled cells within cortical cells called microsclerotia (Jumpponen and Trappe 1998). Mycorrhizal fungi positively affect cycling of macronutrients because they mobilise phosphorus and nitrogen, and are an important carbon sink in the soil (Bonfante and Genre 2010, Johnson et al. 2015). AM fungi are the most widespread fungal symbionts of plants, belonging to the phylum Glomeromycota (Smith and Read 2008). AM fungi form symbiotic relations with plants that have multiple benefits for both partners. In exchange for oxygen and organic carbon, AM fungi enhance plant tolerance to variable water conditions, protect plants against toxic metals and pathogens, and improve their nutrient acquisition (Jacott et al. 2017, Idoia et al. 2004). Therefore, mycorrhizal plants often exhibit improved water status in comparison with non-mycorrhizal plants, which is thought to be a consequence of their greater plant growth (Augé 2004). In the case of carbon limitation, it has been shown that the symbiotic associations become stronger rather than weaker, which suggests that the support of mycorrhizal fungi is of high priority for stressed plants (Gange and West 1994, Ayres et al. 2006, Hartley and Gange 2009, Fontana et al. 2009, Zhang et al. 2015).

Many plant species colonising wetlands exhibit an amphibious character, therefore they can thrive both in water and on dry land, where they develop structurally and/or functionally different growth forms (Gaberščik 1993, Germ and Gaberščik 2003, Boeger and Poulson 2003, Klančnik et al. 2014). Studies of fungal colonisation carried out in amphibious species from an intermittent lake have revealed that the frequency of fungal colonisation may differ significantly among plant specimens of the same species when growing under contrasting water regimes (Šraj et al. 2006, Dolinar et al. 2010, 2016, Gaberščik et al. 2017). However, very little is known about the seasonal dynamics and functionality of these root fungal structures in amphibious plant species that are subjected to seasonal changes of water level in their habitats (Miller 2000, Šraj et al. 2006).

Tall plantain, *Plantago altissima* L., is an amphibious species that can sustain moderate water level fluctuations (Martinčič and Leskovar 2002). We aimed to investigate the seasonal dynamics of fungal colonisation of roots of this species growing in the area of intermittent Lake Cerknica, subjected to frequent water level fluctuations during the vegetation season. We investigated the presence and abundance of AM fungal structures and dark-septate-endophyte fungal structures. To evaluate the possible interactions between fungi and plant conditions, we also examined growth parameters. We hypothesised that the presence of surface water in the habitat of tall plantain negatively affects plant root colonisation with AM fungi and positively affects the occurrence of dark-septate-endophyte fungal structures that mitigate stress conditions, and that the level of fungal colonisation during the season is related to growth parameters and plant phenology.

Material and methods

Study site

The study was performed in the area of intermittent Lake Cerknica. Lake Cerknica is situated at the bottom of the karst polje Cerknica Polje (38 km^2).

Due to abundant precipitation in spring and autumn and occasionally also in other seasons, Cerknica Polje changes into a lake that has an average size of 20–25 km^2. Floods last on average 260 days a year and the dry period usually starts in late spring (Kranjc 2003). The vegetation pattern shows a clear zonal distribution that depends on the extent and frequency of floods (Martinčič and Leskovar 2003).

Studied species

Plantago altissima L. belongs to the family of plantains (Plantaginaceae). It is a perennial herbaceous plant that blooms from May to July (Martinčič et al. 2007). It is distributed in the south-eastern Europe ranging from Pannonian lowland to northern Italy, where it reaches its western limit. Plant material was sampled at wet meadow at Rešeto region, which is located at the edge of the lake in the vicinity of the village Dolenje Jezero (N 45°46'17" (45.7715°) E 14°21'24" (14.3568°)), at a site where soil was water-saturated and even flooded most of the vegetation season except in September. Wet meadow is habitat of a community *Deschampsio - Plantaginetum altissimae*. Beside *Plantago altissima* and *Dechampsia caespitose,* species like *Sanguisorba officinalis*, *Allium ungulatum*, *Genista tinctoria Gratiola officinalis* and some sedges could be found in the area. The area is flooded part of the year, usually in spring and autumn (Martinčič and Leskovar 2002). The extent and duration of floods affect different plant species in different way. In some species environmental extremes accelerate the senescence processes. Water level fluctuations during the experimental season are presented in Figure 1. The water level measurements took place close to the sampling site in the river Stržen (cca. 500 m far) that meanders through the Cerknica Polje. High water level in the peak season (June) resulted in a die-off of the aboveground organs of *P. altissima* that developed once again after a water level drop in July. The presence of surface water at the sampling location is indicated in Figure 3 by grey symbols. From 10 to 20 plants were sampled randomly four times during the vegetation period.

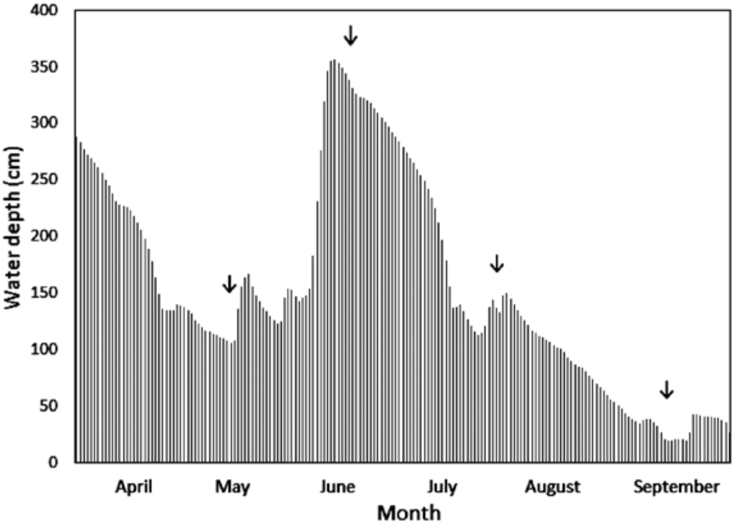

Figure 1: Water level fluctuations measured in the riverbed of Stržen at the weather station Stržen – Dolenje jezero during sampling period (http://vode.arso.gov.si/hidarhiv/pov_arhiv_tab.php). Arrows indicate sampling dates.

Slika 1: Spremembe vodostaja, merjene v koritu Stržen pri meteorološki postaji Stržen - Dolenje jezero v času vzorčenja (http://vode.arso.gov.si/hidarhiv/pov_arhiv_tab.php). Puščice označujejo datume vzorčenja.

Root colonisation with fungi

A block of soil (20 cm × 20 cm × 25 cm) was dug out around the roots of each plant, with the soil and the roots taken from the ground together, and then being transferred to the laboratory. Fine roots were carefully separated from the soil and fixed in ethanol, for the determination of fungal colonisation. These roots fixed in ethanol were later thoroughly washed with distilled water, cleared with 10% KOH, and stained with 0.05% Trypan blue, according to the procedure described by Philips and Hayman (1970). Fungal colonisation of each plant sample was determined on 30 1-cm-long fine-root segments, according to Trouvelot et al. (1986). The root segments were examined under a microscope (Olympus CX41; Olympus, Hamburg, Germany) and photographed using a CCD camera (Colourview II; Soft Imaging System, Münster, Germany) using the Cell Olympus Imaging software. Root segments were ranked into six classes of mycorrhizal colonisation [%] (0, <1, 1−10, 11−50, 51−90, >90) (Trouvelot et al. 1986). The factors determined were: mycorrhizal frequency (proportion of infected root segments by any fungal structure); mycorrhizal intensity (abundance of mycorrhizal colonisation for each root segment); and abundance of different arbuscular-mycorrhizal and dark-septate-endophyte fungal structures, namely arbuscules, vesicles, hyphal coils, and microsclerotia per sample. In addition to the above-mentioned structures, mycorrhizal frequency also comprised the frequency of fungal hyphae.

Growth analysis

We performed growth analysis of whole plants. For each of the 10 to 20 plants per sampling site, we determined fresh and dry mass of below- and aboveground organs, number of flowers, length, width, dry mass of leaves, number of leaves per plant, and plant and leaf water contents.

Statistical analyses

Differences in plant traits including colonisation of roots with fungi were tested using ANOVA or non-parametric Kruskal–Wallis tests with Bonferroni's correction. Normal distribution was tested using the Shapiro-Wilk test. SPSS Statistics 19.0 was used for calculations. To find possible relationships between measured parameters, Spearman's rank correlation analysis was performed (IBM SPSS Statistics 19). Redundancy analysis (RDA) was used to determine whether variations in response variables were related to explanatory variables using CANOCO for Windows 4.0. Monte Carlo permutation tests were carried out to test the significance of the relationships between fungal and plant parameters. The level of significance was accepted at $p \leq 0.05$.

Results

Plant traits

In May, when the majority of plants were still in the vegetative phase, leaf width and length differed considerably from the other three samplings, while they were more or less the same in June, July and September (Table 1). Inflorescences have become very numerous in June, while only a few buds had already been present in May. Belowground biomass was at the same level until September when leaf biomass along with belowground biomass both reached peak values. The lowest values of leaf and plant water content were recorded in September. Mycorrhizal intensity was highest in July and dropped significantly in September.

The overall frequency of fungal structures (including hyphae) was relatively high, ranging from 50% to 90% (Figure 2, upper graph). In plants from the vegetative phase, we observed similar levels of mycorrhizal frequency as during the flowering phase, whereas it significantly decreased during the senescence phase in September. The number of vesicles as storage organs and arbuscules, which indicate active mycorrhiza, was significantly higher during the flowering phase and declined towards the end of the season (Figure 2, lower graph). Similar trend was observed for microsclerotia density, which was significantly reduced in September.

Table 1: Plant growth parameters, plant and leaf water content, and mycorrhizal intensity in *Plantago altissima* in different phases of the vegetative season. Different letters indicate differences in measured parameters among different months ($p<0.05$).

Tabela 1: Parametri rasti rastlin, vsebnost vode v rastlinah in listih ter obseg mikorize pri visokem trpotcu v različnem času vegetacijskega obdobja. Različne črke označujejo razlike med merjenimi parametri v različnih mesecih ($p<0,05$).

	Month of sampling			
Parameter	May	June	July	September
Number of buds/plant	0.25±0.42^{b}	1.2±0.49^{c}	0^{a}	0^{a}
Number of inflorescences/plant	0^{a}	7.5±7.6^{b}	0^{a}	0^{a}
Number of leaves/plant	6.1±2.0^{ab}	4.4±1.7^{a}	6.8±2.6^{b}	4.9±1.0^{ab}
Average leaf length (cm)	12.6±1.7^{c}	27.1±3.2^{b}	21.4±2.3^{a}	28.4±1.8^{ab}
Average leaf width (cm)	0.79±0.23^{b}	1.39±0.21^{a}	1.20±0.22^{a}	1.27±0.2^{a}
Leaf dry mass /plant (g)	1.16±0.66^{b}	1.91±0.65^{a}	1.84±1.09^{a}	3.53±0.86^{a}
Belowground dry mass/plant (g)	1.04±0.76^{a}	0.82±0.33^{a}	1.05±0.81^{a}	1.74±0.76^{b}
Water content (whole plant) (%)	0.60±0.10^{ab}	0.64±0.06^{a}	0.63±0.04^{ab}	0.58±0.06^{b}
Leaf water content (%)	0.67±0.06^{ab}	0.73±0.04^{a}	0.66±0.03^{b}	0.47±0.06^{c}
Mycorrhizal intensity (M%)	6.63±2.67^{ab}	7.95±1.06^{a}	10.01±4.13^{b}	3.73±1.69^{c}

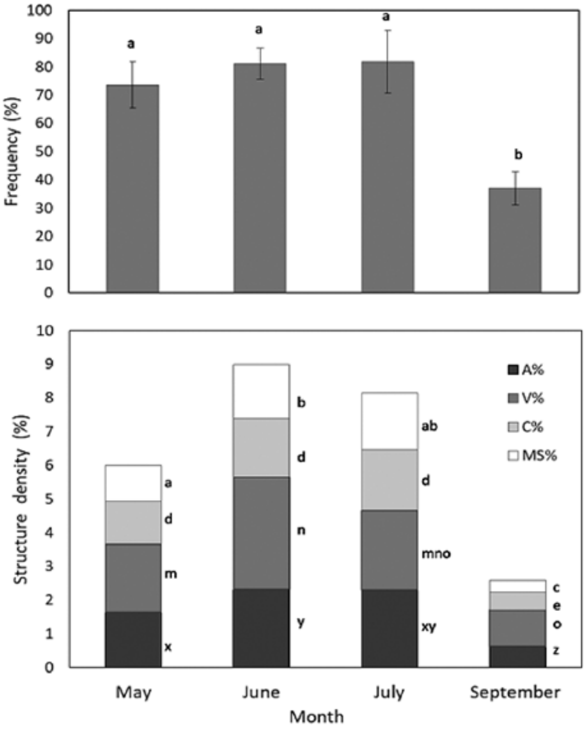

Figure 2: The frequency (upper graph) and density (lower graph) of different fungal structures, i.e., arbuscules (A%), vesicles (V%), hyphal coils (C%), and microsclerotia (MS%) in *Plantago altissima* roots in different phases of the vegetative season. Different letters indicate differences in measured parameters among different months ($p<0.05$).

Slika 2: Pogostost (zgornji graf) in gostota (spodnji graf) različnih glivnih struktur, tj. arbuskulov (A%), veziklov (V%), svitkov (C%), in mikrosklerocijev (MS%) v koreninah visokega trpotca v različnem času vegetacijskega obdobja. Različne črke označujejo razlike med merjenimi parametri v različnih mesecih ($p<0{,}05$).

Relations between fungal and leaf parameters

Plant water content was positively related to the density of fungal structures ($p<0.05$),. The correlation coefficients were 0.35 for arbuscules, 0.46 for vesicles, 0.39 for coils and 0.46 for microsclerotia, respectively. Belowground dry mass wasnegatively related to mycorrhizal frequency (R^2= -0.33; $p<0.05$). The linear regression between leaf water content and overall mycorrhizal frequency was shows positive relation to ($r^2 = 0.56$; $p<0.05$) (Figure 3).

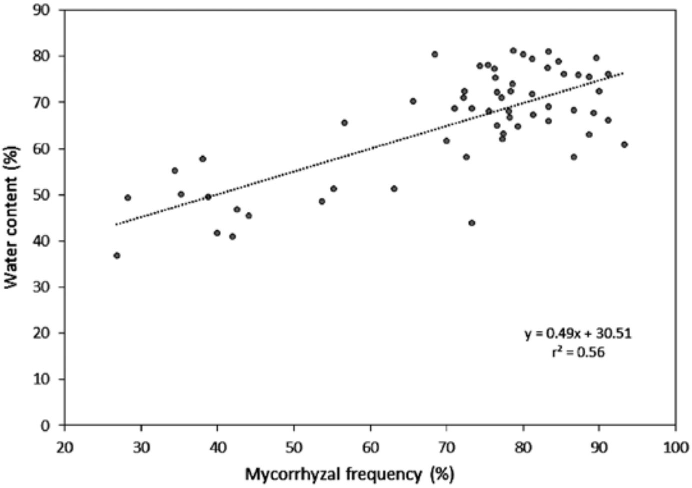

Figure 3: The linear regression of leaf water content and mycorrhizal frequency in *Plantago altissima.* (n=60; $p<0.05$)

Slika 3: Regresija vsebnosti vode v listih in pogostosti mikorize pri visokem trpotcu (n=60; p<0,05).

Redundancy analysis that was run to explain the variability of growth parameters with fungal structures revealed that out of all fungal parameters, only mycorrhizal frequency revealed to be significant. This parameter explained only 15% of the variability of growth parameters. However, when redundancy analysis was run to explain the variability of fungal structures abundance with growth parameters, it showed that altogether these parameters explained 33% of the variability of fungal structures frequency. Mycorrhizal frequency was positively related to leaf water content and negatively to morphometric leaf parameters, namely leaf length and width, leaf water content, number of leaves, and leaf dry mass (Fig. 3). The density of specific structures per infected segments was negatively related to growth parameters as well as to leaf water content.

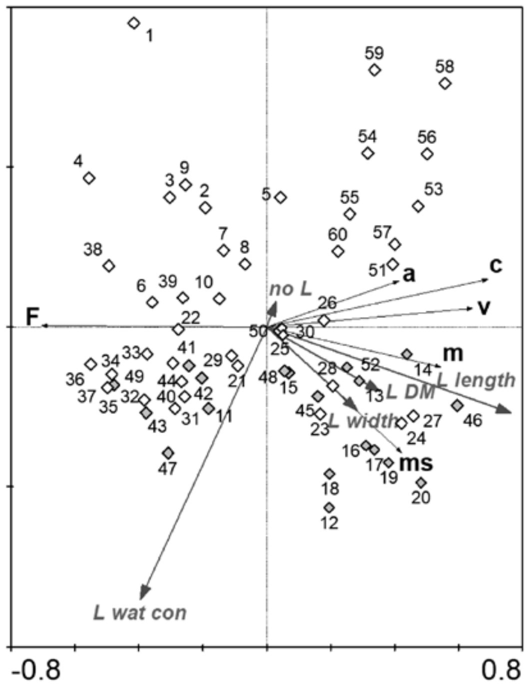

Figure 4: Redundancy analysis plot showing the relationship between leaf properties and mycorrhizal frequency (F), and density of arbuscules (a), vesicles (v), microsclerotia (ms), and coils (c) per colonised root segment in *Plantago altissima* roots; May (1-20), June (21-40), July (41-50), and September (51-60); open symbols indicate samples growing at locations without surface water during the sampling, while grey symbols indicate flooded locations at the time of sampling. Growth parameters: no L – number of leaves, L length – leaf length, L DM – leaf dry mass, L width – leaf width, L wat con – leaf water content.

Slika 4: Graf analize redundance, ki prikazuje razmerje med lastnostmi listov in mikorizno frekvenco (F) ter gostoto arbuskulov (a), veziklov (v), mikrosklerocijev (ms) in svitkov (c) na koloniziranem segmentu korenin visokega trpotca; maj (1-20), junij (21-40), julij (41-50) in september (51-60); odprti simboli označujejo vzorce, ki rastejo na lokacijah brez površinske vode v času vzorčenja, medtem ko sivi simboli nakazujejo poplavljene lokacije v času vzorčenja. Parametri rasti: no L - število listov, L length - dolžina listov, L DM - listna suha masa, L width - širina listov, L wat - vsebnost vode v listih.

Discussion

Plant habitats in Lake Cerknica are permanently exposed to disturbances due to water level fluctuations that can damage the present vegetation (Gaberščik et al. 2003). New colonisation of disturbed area with plants needs efficient strategies of local vegetation. Asmelash et al. (2016) showed that AM fungi play important role in plant community succession, since they increase above- and belowground biodiversity, even though their effect on primary productivity may be negligible. In *Plantago altissima*, the overall frequency of fungal structures was relatively high, ranging from 50% to 90%, while specific AM structures were somewhat less abundant. According to the stress gradient hypothesis (Brooker et al. 2008), the strength of mutualism between symbiotically associated organisms increases as habitat stress conditions worsen (Zhang et al. 2015).

Water level fluctuations affect mineralisation rate and nutrient release as shown by different studies (Boulton and Brock 1999). This is also the case in Lake Cerknica, where the availability of nutrients is low during dry period, while it increases during wetting (Urbanc-Berčič and Gaberščik 2001). The interaction between water and nutrient availability could therefore be crucial in determining the extent of mycorrhizal symbiosis between some wetland plant species (Cornwell et al. 2001). Seasonal dynamics of the frequency of fungal structures in *Plantago altissima* showed high level with increasing trend throughout the season with the exception of September. In September, the frequency significantly decreased, possibly due to the senescence processes and abundant storage in plant rhizomes. A stable level of fungal colonisation throughout the season, irrespective of the hydrological conditions, is probably the result of the preservation of fungal structures that developed in a favourable environment even in adverse conditions (Miller and Sharitz 2000).

The frequency of the majority of fungal structures became more abundant in June and July. In June, plants were in the flowering phase that increased the need for nutrients, as also shown in other studies (Naveed et al. 2012, Dolinar et al. 2016). Regvar et al. (2006) report that in some plant species active AM can be limited to specific periods of plant ontogenesis, especially to the fruiting and seeding period. In *Plantago altissima*, new assimilation areas developed in July, since deep water from the previous month caused the decay of aboveground structures, including of the assimilation areas.

AM colonization affects a number of plant and soil characteristics, among which the effect on plant water relations is very important (Augé 2004). In our study, plant water content was positively related to the density of the majority of fungal structures, with the exception of arbuscules and coils.

DSE are common plant root colonizers in extreme environments. The hyphae of DSE fungi produce melanin in their cell walls, which increases the toughness of cell walls, increases plant tolerance to pathogens, and protects against dehydration (Jumpponen 2001). This is of great importance in water-fluctuating habitats, however in the case of *Plantago altissima* in Lake Cerknica, microsclerotia density showed similar dynamics over the vegetative season as AM structures, which revealed the potential to increase resources availability in studied plant species..

Summary

Intermittent wetlands are characterised by extreme water level fluctuations that affect habitat conditions in time and space. Studies of fungal colonisation carried out in amphibious plant species in an intermittent lake revealed that the frequency of fungal colonisation differs significantly among plant specimens growing under contrasting water regimes. We hypothesized that the presence of surface water in the habitat of tall plantain (*Plantago altissima* L.) negatively affects plant root colonisation with AM fungi, and positively affects the occurrence of dark-septate-endophyte fungal structures that mitigate stress conditions, and that the level of fungal colonisation during the season is related to growth parameters and plant phenology. We investigated the presence and abundance of AM fungal structures and dark-septate-endophyte fungal structures. To evaluate the possible interactions between fungi and plant conditions, we also examined growth parameters. In May, when the majority of plants were still in the vegetative phase, leaf width and length differed the most from the other three samplings (Table 1). Inflorescences have become numerous in June, while only a few buds had already been present already in May. The decay of aboveground organs due to high floods in peak season was followed by new growth of leaves and in the following month the level of fungal colonisation remained the same. Belowground biomass was also at the same level until September when leaf biomass along with belowground biomass both reached peak values. Leaf and plant water content were lowest in September. Mycorrhizal intensity was highest in July and dropped significantly in September. The total frequency of fungal structures (including hyphae) was relatively high, ranging from 50 % to 90 %. In plants from the vegetative phase, we observed similar levels of mycorrhizal frequency as during flowering, whereas it significantly decreased during the senescence phase in September. The number of vesicles increased significantly during

the flowering phase and declined towards the end of the season. Similar trend was observed for DSE structures, namely microsclerotia, as their number was significantly reduced in September. However, the frequency of DSE structures microsclerotia was significantly positively related to AM structures that was not in accordance with our hypothesis. Plant water content was positively related to the density of different fungal structures. Leaf water content was positively related to the mycorrhizal frequency and to the density of specific fungal structures. Belowground dry mass was negatively related to mycorrhizal frequency. All these data revealed the importance of fungal colonisation for plant water status.

Povzetek

Presihajoča mokrišča zaznamujejo skrajna nihanja ravni vodostaja, ki vplivajo na habitate v času in prostoru. Raziskave glivne kolonizacije amfibijskih rastlinskih vrst v presihajočih ekosistemih so pokazale, da se pogostnost glivne kolonizacije znatno razlikuje med rastlinami, ki rastejo različnih vodnih razmerah. Predpostavili smo, da prisotnost površinske vode v habitatu visokega trpotca (*Plantago altissima* L.) negativno vpliva na kolonizacijo rastlinskih korenin z AM glivami in pozitivno na pojavljanje struktur temnih septiranih endofitov, ki blažijo stresne razmere, ter da je obseg glivne kolonizacije med sezono povezan s parametri rasti in fenologijo rastlin. Raziskovali smo prisotnost in obseg AM glivnih struktur ter struktur temnih septatnih endofitov. Za oceno morebitnih interakcij med glivami in rastlinskimi parametri smo preučili tudi parametre rasti. Maja, ko je bila večina rastlin še vedno v vegetativni fazi, so se širine in dolžine listov najbolj razlikovale od drugih vzorcev. Cvetovi so se pojavili junija. Zaradi obsežnih poplav na višku sezone, je prišlo do propada nadzemnih organov in ob upadu vode rasti novih listov, kljub temu pa je raven glivne kolonizacije korenin ostala podobna. Tudi podzemna biomasa je ostala na isti ravni do septembra, ko sta biomasa listov in podzemna biomasa dosegli svoje najvišje vrednosti. Vsebnost vode v listih je bila najnižja v septembru. Intenzivnost mikorize je bila najvišja v juliju in je občutno upadla v septembru. Pogostnost vseh glivnih struktur (vključno s hifami) je bila relativno visoka, saj je segala od 50 % do 90 %. Pri rastlinah v vegetativni fazi smo opazili podobno raven pogostnosti mikorize kot med cvetenjem, medtem ko se je v septembrskem obdobju znatno zmanjšala. V času cvetenja se je število veziklov znatno povečalo in se zmanjšalo proti koncu sezone. Podoben trend smo opazili tudi pri mikrosklerocijih, strukturah DSE, katerih pogostnost se je septembra bistveno zmanjšala. Frekvenca struktur DSE je bila v pozitivni povezavi z AM strukturami, kar ni bilo v skladu z našo hipotezo. Vsebnost vode v rastlinskem tkivu je bila pozitivno povezana z gostoto večine glivnih struktur, razen z arbuskuli in svitki. Suha masa podzemnih organov je bila negativno povezana s pogostnostjo mikorize. Rezultati potrjujejo pomen glivne kolonizacije za rastline v presihajočih mokriščih.

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