Mycorrhizal symbiosis alleviates plant water deficit within and across plant generations via plasticity

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Total word count (excluding summary, references and legends):	5768	No. of Figures:	3
Abstract:	201	No. of Tables:	0
Introduction:	1092	No. of Supporting Information files:	9 (Fig. S1-S6, Table S1-S3)
Materials and Methods:	2029		
Results:	868		
Discussion:	1987		
Acknowledgments:	73		

1 **Abstract**

- 2 • Phenotypic plasticity is essential for organisms to adapt to local ecological
3 conditions. It is expected that mutualistic interactions, such as arbuscular
4 mycorrhizal (AM) symbiosis, mediate plant phenotypic plasticity, although it is not
5 clear to what extent this plasticity may be heritable (i.e., transgenerational
6 plasticity).

- 7 • We tested for plant plasticity within- and across-generations in response to AM
8 symbiosis and varying water availability in a full factorial experiment over two
9 generations, using a genetically uniform line of a perennial apomictic herb,
10 *Taraxacum brevicorniculatum*. We examined changes in phenotype, performance,
11 and AM fungal colonization of the offspring throughout plant development.

- 12 • AM symbiosis and water availability triggered phenotypic changes during the life
13 cycle of plants. Additionally, both triggered adaptive transgenerational effects,
14 especially detectable during the juvenile stage of the offspring. Water deficit and
15 absence of AM fungi caused concordant plant phenotypic modifications towards a
16 “stress-coping phenotype”, both within- and across-generations. AM fungal
17 colonization of offspring was also affected by the parental environment.

- 18 • AM symbiosis can trigger within-generation and transgenerational plasticity in
19 terms of functional traits related to resource-use acquisition and AM fungal
20 colonization. Thus, transgenerational effects of mycorrhizal symbiosis are not
21 limited to plant fitness, but also improve plants’ ability to cope with environmental
22 stress.

23 **Key words:**

24 Adaptation, Arbuscular mycorrhizal symbiosis, drought stress, transgenerational effects,
25 functional traits, AM fungal colonization, phenotypic plasticity

26 **Introduction**

27 Abiotic environment and prevailing biotic interactions select for the best-adapted
 28 individuals within and across species in terms of their functional traits (de Bello et al.,
 29 2012; Vellend, 2016). The probability of an individual to withstand this selection may
 30 depend on the nature and severity of the environmental condition, the heritable genetic
 31 variability, but also on its phenotypic plasticity (Price, Qvarnström, & Irwin, 2003).
 32 Phenotypic plasticity is the ability of an organism to modify its performance and trait
 33 expression in response to the environment without altering the DNA sequence (Price et
 34 al., 2003). These changes could operate within the life cycle of the organism subjected
 35 to those conditions (“within-generation plasticity”). Additionally, they may be
 36 transmitted to the following generations via transgenerational effects (also called
 37 “across-generation or transgenerational plasticity”), meaning that the abiotic and biotic
 38 environment experienced by the parental generation influence the phenotype of the
 39 offspring (Herman & Sultan, 2011; Jablonka & Raz, 2009). Recent studies suggest that
 40 transgenerational effects could play a key role in the adaptive response of organisms to
 41 stressors, proven particularly essential during the juvenile stages (Dechaine, Brock, &
 42 Weinig, 2015; Lämke & Bäurle, 2017; Latzel, Janeček, Doležal, Klimešová, &
 43 Bossdorf, 2014; Puy, Carmona, Dvořáková, Latzel, & de Bello, 2020). While the effect
 44 of abiotic conditions on transgenerational plasticity has been repeatedly demonstrated,
 45 little is known about the relative effect of transgenerational effects triggered by biotic
 46 conditions (Alonso, Ramos-Cruz, & Becker, 2019; Puy, de Bello, et al., 2020), and even
 47 less about how they interact with abiotic factors.

48 Together with species’ adaptations to environmental conditions in a site, biotic
 49 interactions are considered key drivers of plant community assembly (de Bello et al.,
 50 2012). Among these, positive interactions such as mycorrhizal symbiosis are essential in
 51 determining, and potentially expanding, the realized niches of species (Gerz, Bueno,
 52 Ozinga, Zobel, & Moora, 2018; Peay, 2016; van der Heijden, Wiemken, & Sanders,
 53 2003). Arbuscular mycorrhizal (AM) symbiosis is a widespread mutualistic association
 54 between plant roots and fungi (from the subphylum Glomeromycotina; Spatafora et al.,
 55 2016). This association is considered mutually beneficial, since, in exchange for
 56 photosynthetic carbon, the AM fungi provide host plants with soil nutrients (mainly
 57 phosphates; Smith & Read, 2008), mitigate abiotic stress (e.g. making the host more
 58 tolerant to drought; Aroca, Porcel, & Ruiz-Lozano, 2012; Augé, Toler, & Saxton, 2015;

59 Doubková, Vlasáková, & Sudová, 2013), and increase resistance to biotic stress,
60 including pathogens (Pozo, López-Ráez, Azcón-Aguilar, & García-Garrido, 2015;
61 Smith & Read, 2008). Besides the mutual effects of the AM interaction, the occurrence,
62 abundance, activity, and the final outcome of the interaction (from positive to negative)
63 are known to be affected by multiple factors (Hoeksema et al., 2010; N. C. Johnson,
64 Graham, & Smith, 1997). These factors include intrinsic drivers such as the genotype of
65 both partners, or the plant developmental stage or sex (Jones & Smith, 2004; Varga &
66 Kytöviita, 2010). Also, external drivers such as the biotic environment (Šmilauer,
67 Košnar, Kotlínek, & Šmilauerová, 2020) and/or soil nutrient and water availability
68 (Martínez-García, de Dios Miranda, & Pugnaire, 2012; Pozo et al., 2015) might be
69 important. Phosphorus, nitrogen or water deficiency in plants generally, at least when
70 light is not limiting, stimulates AM symbiosis and influences the abundance of AM
71 structures (i.e. arbuscules, vesicles, etc.; Martínez-García et al., 2012; Pozo et al., 2015).
72 However, it remains unclear whether the environmental conditions experienced in
73 parental generations could affect the functioning of AM symbiosis of the offspring
74 generation (De Long et al., 2019).

75 As illustrated before, the fitness benefits of plants in AM symbiosis are
76 relatively well known (Lu & Koide, 1994; Smith & Read, 2008). However, it is unclear
77 whether these benefits partly operate through phenotypic plasticity induced by the
78 interaction (Vannier, Mony, Bittebière, & Vandenkoornhuyse, 2015). AM symbiosis
79 may lead to adaptive changes of plant morphological traits such as modifications in root
80 architecture (Fusconi, 2014; Goh, Veliz Vallejos, Nicotra, & Mathesius, 2013; Nuortila,
81 Kytöviita, & Tuomi, 2004) or in traits of the so called “plant economic spectrum”. Since
82 these traits are associated with a fundamental trade-off between organisms along a
83 resource-use-acquisition vs. resource-use-conservation gradient, they could be used to
84 describe the plant resource-use strategy (Díaz et al., 2016). Moreover, it also remains
85 unclear whether AM symbiosis in a parental generation can trigger similar or different
86 phenotypic changes in the offspring (i.e. transgenerational effects) and whether these
87 changes are beneficial (Koide, 2010; Varga, Vega-Frutos, & Kytöviita, 2013). As such,
88 it is crucial to assess the relative effect of combined biotic and abiotic drivers on within-
89 generation and transgenerational plasticity, as biotic drivers can potentially modulate
90 the effect of abiotic stress (González, Dumalasová, Rosenthal, Skuhrovec, & Latzel,
91 2017; Metz, von Oppen, & Tielbörger, 2015).

It should be noted that transgenerational effects can be due to two mutually non-exclusive mechanisms: environmentally-induced heritable epigenetic modifications in the offspring (Lämke & Bäurle, 2017), or differences in seed provisioning, seed nutritional quality or hormonal balance provided by the maternal plants in the embryos (Dechaine et al., 2015; Germain, Grainger, Jones, & Gilbert, 2019; Herman & Sultan, 2011). In comparison, transgenerational effects originated from embryo modifications play more significant role during early stages of the development, but they tend to fade away with time (Latzel, Klimešová, Hájek, Gómez, & Šmilauer, 2010). Most of the existing evidence demonstrates that having mycorrhizal parents can be beneficial during the early stages of development of the offspring, i.e. increasing biomass, survival, growth rate, nutrient content, and seed production (Heppell, Shumway, & Koide, 1998; Koide, 2010; Varga, 2010; Varga et al., 2013). However, when testing for transgenerational effects of AM symbiosis, both mechanisms have rarely been considered (but see Varga et al. (2013) where seed mass was used as a covariate). Further, Varga & Soulsbury (2017, 2019) have investigated the potential epigenetic mechanism (i.e., DNA methylation) governing the transgenerational effects of AM symbiosis. **Error! Bookmark not defined.** Nevertheless, it has not yet been tested whether the transgenerational effects of AM symbiosis persist further into adult stage, neither whether these effects influence plant functional traits nor AM fungal colonization of the offspring.

We conducted a two-generation experiment to test for within- and across-generation plant plasticity in response to AM symbiosis using the perennial apomictic herb *Taraxacum brevicorniculatum*. Further, in order to test whether both plasticity types interact with abiotic stress conditions, we included a watering regime treatment. We then tested whether within- and across-generation plant plasticity were adaptive, e.g., resulting in an improved ability of the offspring to cope with water limitation. Additionally, we evaluated the persistence of the transgenerational effects throughout offspring development by measuring phenotypic traits, performance, and AM fungal colonization on juvenile and adult offspring.

124

125 **Materials and Methods**

126

127 *Study material*

128 *Taraxacum brevicorniculatum* Korol. is an obligate apomictic polycarpic perennial
 129 plant (Kirschner, Štěpánek, Černý, De Heer, & van Dijk, 2013). Like most species of
 130 the genus *Taraxacum*, it has a wide ecological niche, accepting all types of soils, pH
 131 and moisture levels (Luo & Cardina, 2012), and forms an active symbiosis with AM
 132 fungi (J. Puy, personal obs. based on a preliminary study). In this study we used
 133 genetically identical seeds collected from a population of plants grown under the same
 134 glasshouse conditions for several generations (collected and genetically identified by
 135 Kirschner *et al.* 2013). This strategy ensured homogeneous genetic and epigenetic
 136 variation in the plant material. Since *T. brevicorniculatum* is an obligate apomictic
 137 species, all seeds produced by a plant are effectively clones, thus enabling the study of
 138 plasticity within and across generations (Puy *et al.*, 2018). In other words, all plants in
 139 the experiments were genetically identical, and after experiencing different conditions
 140 during the parental generation, the offspring only differed in non-genetic (i.e., non-
 141 genetic or epigenetic effects) inherited information.

142

143 *Experimental setup*

144 **Parental generation.** In order to induce the potential transgenerational effects related to
 145 mycorrhizal symbiosis and water availability, we first conducted an experiment in
 146 which the parental generation was grown under different conditions in a glasshouse for
 147 three months (April-July 2017). We grew 364 genetically identical individuals of *T.*
 148 *brevicorniculatum* in individual pots (7 x 7 x 18 cm), half inoculated with AM fungi
 149 (AM) and the other half without (NM). In addition, half of these individuals were grown
 150 with sufficient water and half under a water deficit scenario, see below for details.

151 The substrate consisted of 2:1 mixture of sterilized sand and natural soil
 152 collected from a mesic meadow 30 km southeast of Tabor, 660 m a.s.l. (Vysočina
 153 region, Czech Republic, 49.331N, 15.003E) where *Taraxacum sect. Ruderalia* was

154 present. The natural soil was firstly sieved using a 4 mm mesh sized sieve to remove
155 any stones, macrofauna, or rhizomes from the soil. For the AM treatment the natural
156 soil containing indigenous microbial community was used; whereas for the NM
157 treatment the same soil was sterilized via γ irradiation (>25kGy dose; McNamara,
158 Black, Beresford, & Parekh, 2003). To compensate for the loss of other soil microbes
159 due to the sterilization, a microbial wash was also added (Liang et al., 2015). We
160 obtained the microbial wash by blending 5kg of non-sterilized soil in 10 l water and
161 filtering the solution through 20 μ m pore-size filter paper (Whatman® quantitative filter
162 paper, Grade 41) broadly following van der Heijden *et al.*, (1998). Gamma-sterilization
163 did not change neither the C nor the N chemical composition of the soil compared to the
164 non-sterilized soil (Fig. S1).

165 The AM and NM treatments were factorially combined with two levels of water
166 availability. Half of the individuals were subjected to cycles of water deficit (simulating
167 drought stress; W-), while the other half were watered regularly from the bottom
168 ensuring the pot surface was always wet (control; W+). The water deficit treatment
169 included periodic exclusion of watering until when 50% of the individuals had wilted
170 leaves followed by one-week recovery in control conditions. By the end of the
171 experiment, the water deficit treatment comprised two water deficit pulses (the first
172 started 12th of May and the second 15th of June) that lasted three weeks plus one-week
173 recovery each.

174 Prior to the establishment of the experiment, seeds were surface sterilised by
175 immersion in 0.5% sodium hypochlorite solution (commercial bleach) for 20 minutes to
176 avoid inoculation via seeds, and then germinated in Petri dishes. After 10 days of
177 germination, the seedlings were transplanted individually into the pots specified above,
178 with 91 replicates per treatment. After three months we harvested all the plants except
179 15 individuals from each of the four treatment combinations (AM W+, AM W-, NM
180 W+, NM W-). These plants were kept for four more months in ambient conditions
181 (water control condition), with a 12 h (20°C) / 12 h (10°C) light/darkness-and-
182 temperature regime and with addition of fertilizer (Kristalon; NPK 15–5–30 + 3Mg +
183 5S) at the concentration of 300 ppm once per month, in order to promote seed
184 production. Relocating the plants to more benign conditions was required to promote
185 seed production especially for the non-mycorrhizal plants which, at the time of harvest,

186 had not flowered yet. Then, seeds of each plant were collected, and after measuring the
187 average seed mass per plant, were stored in cold (2-4 °C).

188 **Offspring experiment.** A similar glasshouse experiment to the one described above
189 was repeated the following year (April-August 2018) with the seeds produced by the
190 parental generation in the first experiment. The aim of the offspring experiment was to
191 test for adaptive transgenerational effects of AM symbiosis and water availability on the
192 offspring at their juvenile and the adult stages. We tested this with a full factorial design
193 where the offspring plants from each of the four parental treatments were exposed again
194 to the four possible conditions (AM W+, AM W-, NM W+, NM W-). Thus, the
195 offspring experimental design resulted in 16 combinations: two parental mycorrhizal
196 inoculations (Par. M: AM/NM) x two parental water availability levels (Par. W: W+/W-
197) x two offspring mycorrhizal fungal inoculations (Off. M: AM/NM) x two offspring
198 water availability levels (Off. W: W+/W-). Since the seed mass of AM parents was on
199 average lower than that of NM parents (see below *Results of parental generation*, Table
200 S1), and seed provisioning could be a potential mechanism of transgenerational effects
201 (Herman & Sultan, 2011). We controlled for it by classifying seeds from all parental
202 treatments into 5 size categories. Then, we took the same number of seeds from each
203 size-group in each parental treatment, resulting in a similar distribution of seed sizes
204 between parental treatments. Thus, the offspring experimental design finally resulted in
205 the 16 combinations x 5 seed size categories x 8 seedlings = 640 pots (Fig. S2).

206 Plants were harvested at two different developmental stages. Half of the
207 offspring plants were harvested 1.5 months after planting, at their juvenile stage; and the
208 rest of the replicants were harvested five months after planting, at their adult stage
209 before they flowered. Pots, substrate and watering regime were the same as in the
210 parental experiment to ensure the most similar conditions. However, the first water
211 deficit pulse in the offspring generation lasted four weeks instead of three (first one
212 started the 25th of April and the second one, the 1st of June) to ensure comparable effects
213 on plants physiology (i.e., percentage of plants with wilted leaves). To facilitate the
214 application of the treatments, four replicates of a parental treatment were placed in
215 parallel, one in each offspring treatment (Fig. S2).

216

217

218

219 *Measured traits*

220 We measured a set of important plant traits in both generations. For each plant in the
221 parental generation we measured survival, total dry biomass (aerial plus root biomass),
222 seed output (i.e., number of seeds) and seed mass at the time of harvest. For five
223 randomly chosen plants per treatment, we measured C, N and P content of the seeds,
224 considered to be reliable indicators of seed reserve materials (Toorop et al., 2012). Total
225 C and N content were determined by dry combustion using an elemental analyser
226 (CHNS Elemental Analyzer vario MICRO cube, Elementar Analysensysteme GmbH,
227 Germany). Total P was determined by flow injection analysis.

228 Additionally, we measured several above- and belowground vegetative plant traits. For
229 each plant, two leaves were scanned for leaf area and weighed for fresh mass and dry
230 mass after drying at 60° C (48h) to estimate specific leaf area (SLA; leaf area per dry
231 mass, mm²/mg) and leaf dry matter content (LDMC; leaf dry mass per leaf fresh mass,
232 mg/mg). Roots were carefully extracted, washed and a subsample of roots (6 cm²) was
233 scanned at 600 dpi with an Epson Perfection 4990 scanner. Total root length, average
234 root diameter (mm), and distribution of root length in different diameter classes were
235 determined by using the image analysis software WinRHIZO Pro, 2008 (Regent
236 Instruments Inc., Quebec, Canada). After scanning, the root subsample and the rest of
237 the root system were dried for 48 h at 60 °C and weighed. We used these measurements
238 to estimate specific root length (SRL; root length per dry mass, m/g), and fine roots
239 percentage (root length with a diameter < 0.5mm per total root length). Further, we
240 estimated root biomass allocation (i.e., root mass factor; RMF; root biomass per total
241 biomass, g/g) after drying the remaining radicular part at 60° C (48h).

242 For each plant in the offspring generation, at the time of the respective harvest
243 (i.e., juvenile and adult offspring harvest), we measured total dry biomass (aerial plus
244 root biomass), and the same above- and belowground vegetative traits as described
245 above. Additionally, we analyzed the content of C, N and P in the leaves of two
246 randomly chosen plants from the juvenile stage and eight plants from the adult stage per
247 treatment, following the methods described above. The root subsamples were stained
248 with Chlorazol Black according to the protocol by Šmilauer, Košnar, Kotlínek, &
249 Šmilauerová (2020). We quantified the AM fungal colonization by measuring the

percentage of root length colonized (%RLC) by AM fungal structures (arbuscules, vesicles and hyphae). Magnified intersection method (McGonigle, Miller, Evans, Fairchild, & Swan, 1990) was used with 400 x magnification using a light microscope with graticule inserted into the eyepiece. All the specific structures of AM fungi (arbuscules, vesicles and hyphae) that intersected the vertical line (i.e., root in horizontal position) were counted for at least 100 intersections per root sample. We further calculated the arbuscule:vesicle ratio (relative abundance of arbuscules per vesicles), suggested as an indicator of the fungal activity status and the relative cost or benefit of the fungus to the host plant (Braunberger, Miller, & Peterson, 1991; Titus & Lepš, 2000).

260

Statistical analysis

All analyses were carried out using R v3.2.3 (R Core Team 2016) with $\alpha=0.05$ as significance level. In the parental generation, the effects of the mycorrhizal inoculation (two levels), the water availability (two levels), and their interaction were analysed by using linear effects models. Plant total biomass was always log-transformed.

In the offspring generation, individuals were grouped into sixteen different treatments (as a result of the combination of four factors with two levels each) depending on parental and offspring conditions. Two of the factors corresponded to parental conditions: mycorrhizal fungal inoculation treatment (Par. M: AM/NM), and water availability treatment (Par. W: W+/W-). The other two factors corresponded to offspring conditions: mycorrhizal fungal inoculation treatment (Off. M: AM/NM) and water availability treatment (Off. W: W+/W-). We analysed the effects of parental and offspring conditions on plant traits of the offspring using linear effects models where the four experimental factors (two parental, and two offspring conditions) and all their interactions were used as fixed effects. Additionally, in order to control for differences between parental treatments in seed provisioning or quality provided by the maternal plants (Herman & Sultan, 2011; Toorop et al., 2012), we included seed mass and seed stoichiometry as covariates in the model (i.e. as fixed effects). The seed stoichiometry values were computed assigning the scores of the first axis of a principal components analysis (PCA) that combined the C, N and P chemical composition of the seeds and absorbed 60% of the variation. Seed stoichiometry was not correlated with seed mass

(Pearson correlation = -0.18, $P = 0.44$). Any effect of the parental conditions: either direct (Par. M or Par. W) or in interaction with the offspring conditions (Off. x Par.) that remained significant after removing the linear part of the maternal investment (seed mass and stoichiometry) was considered a transgenerational effect.

For the analysis of the effect of parental and offspring treatments on AM fungal colonization and arbuscule:vesicle ratio of the offspring, we used identical models, but excluding the offspring mycorrhizal inoculation factor (Off. M) from the model due to the lack of AM fungal colonization in the NM plants.

Additionally, we checked for correlations between plant traits and measures of AM fungal colonization to check which plant traits show plasticity in response to AM fungal colonization and to examine whether these changes could partially explain the benefits of AM symbiosis to the host plant, meaning that they are adaptive.

Results

Parental generation

In the parental generation, the water deficit treatment decreased *T. brevicorniculatum* total plant biomass and survival, but only on NM plants, with no effect on AM plants (Fig. S3a,b; Table S1). AM fungal inoculation increased plant growth, survival and reproductive investment (i.e., number of seeds per unit plant biomass), with no effect on the total number of seeds produced per plant (Fig. S3, Table S1). Additionally, seeds of AM plants were lighter than the ones of NM plants, and with higher N, P and C contents, although only the latter one was significant (Fig. S3, Table S1). Nevertheless, the seed macronutrients stoichiometry, i.e. C:N:P ratios, did not differ between AM and NM plants (Fig. S3, Table S1).

Offspring plant traits

In the juvenile offspring, the majority of the measured plant traits were strongly affected by both offspring treatments (offspring mycorrhizal fungal inoculation treatment, Off.

311 M; and offspring water availability treatment, Off. W), except leaf C:N ratio and SRL
312 that were affected only by the mycorrhizal and by the water treatment respectively (Fig.
313 1 and Table S2). In general, plants under water deficit and absence of AM symbiosis
314 were smaller, with higher biomass allocation into the roots (i.e., higher RMF), ticker
315 leaves and roots (lower SLA and SRL), and lower P content and C:N ratio on leaves
316 (Fig. 1 and Table S2).

317 After removing the effect of seed mass and seed stoichiometry (maternal
318 investment effects), we found transgenerational effects (i.e., offspring plants were
319 affected by the parental conditions) in most of the traits (i.e., seven out of nine, Table
320 S2). When comparing the effects of both parental treatments (Par. M and Par. W), we
321 found that parental mycorrhizal inoculation triggered transgenerational effects in all
322 measured plant traits, while the parental water treatment induced changes only on three
323 traits and generally in interaction with the offspring conditions (Fig. 1; Table S2).
324 Juvenile offspring of mycorrhizal parents (Par. M) had in general lower biomass, lower
325 allocation to the roots (lower RMF) but thinner roots (% Fine roots), and higher LDMC
326 and leaf P content (Fig. 1a,b,c,d,g and Table S2). Except for biomass and LDMC, the
327 direction of the response to the parental treatment was concordant with the response to
328 the offspring treatment. For example, mycorrhizal offspring showed lower RMF and
329 more P content on leaves, and these effects were further pronounced when offspring
330 also had mycorrhizal parents (Fig. 1b,d and Table S2). Although in interaction with
331 other factors, we found transgenerational effects induced by the parental water
332 treatment. Mycorrhizal offspring of parents under water deficit (Par. W) increase the
333 SRL and slim down the roots, when also had mycorrhizal parents (Par. W x Off. M x
334 Par. M Fig. 1f,g and Table S2).

335 At the adult stage, the offspring treatments (Off. M and Off. W) were still the
336 main drivers of plant plasticity (Fig. 2 and Table S3) and generally the responses of the
337 traits were in the same direction than those during juvenile stage (leaf P content and
338 total biomass; Fig. 2 and Table S3). Nonetheless, LDMC plasticity in response to
339 offspring conditions (Off. M and Off. W) reversed compared with the juvenile stage.
340 The offspring with water deficit and absence of AM symbiosis had higher LDMC
341 during the juvenile stage, but lower LDMC during the adult stage (Fig. 1b and Fig. 2b).
342 Similar reversed response happened in RMF only when offspring were with water
343 deficit. Also, root traits that did not respond to the offspring treatments during juvenile

stage, started responding during adult stage. At the latter stage, plants with water deficit and non-mycorrhizal plants had thinner and more absorptive roots (higher SRL and % Fine roots; Fig. 2f, g and Table S3). Finally, at this stage, we still detected significant transgenerational effects on plant traits, but only on RMF (Fig. 2b and Table S3). Adult offspring of mycorrhizal parents (Par. M) still had lower allocation to the roots (lower RMF).

350

351 *Offspring AM fungal colonization*

The water availability treatments (Off. W) modified the AM fungal colonization of the juvenile offspring. Plants grown with water deficit cycles had less root length colonized by AM fungi (lower %RLC) but had higher arbuscule:vesicle ratio than control plants (Fig. 3a, b and Table S2). Additionally, AM fungal colonization was affected by the parental treatments only when offspring plants had water deficit (Off. W x Par. W; Table S2). Offspring of parents that did not experience water deficit had higher %RLC than the ones of parents that had experienced water deficit (Fig. 3a; Table S2). For the arbuscule:vesicle ratio we did not detect significant transgenerational effects (Fig. 3b, Table S2 and Fig. S4).

During the adult stage, we found no significant difference in %RLC between the offspring water availability treatments (Off. W), although there was a lower arbuscule:vesicle ratio in plants with water deficit, reversed response compared with what happened during the juvenile stage (Fig. 3c, d, Table S3 and Fig. S4). Also, at this stage we found that AM fungal colonization was affected by the parental treatments only when the offspring plants grew with water deficit cycles. The adult offspring of mycorrhizal parents (Off. W x Par. M) had higher %RLC than the ones of non-mycorrhizal parents (Fig 3c; Table S3).

369

370 **Discussion**

In this study we show the importance of AM symbiosis in triggering phenotypic plasticity in plants, both during their life cycle and in following generations. Such phenotypic changes can improve the ability of individuals to cope with environmental stress and likely increase the species' realized niche. Here we show that mycorrhizal

375 symbiosis could specifically trigger morphological changes related to resource use and
376 resource acquisition strategies within-generations and to succeeding generations.
377 Further, we provide evidence that AM fungal colonization of the offspring could be also
378 affected by parental conditions. Transgenerational effects of mycorrhizal symbiosis and
379 water availability were not caused by differences in the quality and resources provided
380 in the seed (Herman & Sultan, 2011), pointing to heritable epigenetic mechanisms as
381 potential factor transmitting and mediating these effects across generations.

382

383 *Within-generational plasticity on offspring traits is development specific*

384 The strong response of plants of *T. brevicorniculatum* to the conditions experienced
385 during their life cycle (i.e., offspring mycorrhizal fungal inoculation and water
386 availability treatments) shows the high level of plasticity of this plant species. However,
387 we found that the response to these conditions differed in juvenile and adult phases,
388 suggesting specific plant plasticity at different developmental stages (Coleman,
389 McConnaughay, & Ackerly, 1994).

390 As expected, measurements of different fitness-related characteristics (i.e. plant
391 nutrition and growth) suggest that AM symbiosis improved plant performance and
392 mitigated water deficit, since the benefit of being mycorrhizal increased with decreasing
393 water supply (Aroca et al., 2012; Augé et al., 2015; Doubková et al., 2013). First,
394 mycorrhizal fungal inoculation dramatically increased leaf P content at both
395 developmental stages of the offspring (Fig. 1d and Fig. 2d). These results reflect that
396 mycorrhizal plants had better nutritional supply – were provided with important
397 nutrients such as P (Doubková et al., 2013; Lu & Koide, 1994) – despite being in water
398 deficit conditions. Probably because of this, mycorrhizal fungal inoculation also
399 increased growth (i.e., plant biomass) of plant individuals in water deficit conditions to
400 similar levels than growth of non-stress plants. However, this benefit was more
401 pronounced in adult offspring than in juveniles (Fig. 2a vs Fig. 1a), probably due to a
402 greater cost/benefit ratio during the juvenile stage (N. C. Johnson et al., 1997).

403 In both developmental stages, mycorrhizal fungal inoculation and water
404 availability treatments induced significant changes in multiple plant phenotypic traits
405 related to the resource-use strategy of the plant (also called plant economic spectrum;

406 Díaz et al., 2016), including both below- and aboveground traits (Fusconi, 2014; Goh et
407 al., 2013; Nuortila et al., 2004). However, traits seem to respond differently depending
408 on the developmental stage of the plant. Plant phenotypic plasticity triggered by AM
409 fungal inoculation could be either direct (i.e., caused in reaction to AM fungal
410 infection), or indirect by a lack of phenotypic plasticity in reaction to water deficit, since
411 the AM fungi increase the tolerance of host plants to it (Goh et al., 2013; Maherali,
412 2014).

413 During the juvenile stage water availability and mycorrhizal fungal inoculation
414 triggered independent and additive trait plasticity in the same direction (Table S2).
415 Similar to findings of Shumway & Koide (1994), under reduced water availability
416 and/or absence of AM fungi, plants shifted towards more resource-conservative
417 phenotype based on the plant economics spectrum framework (Díaz et al., 2016). This
418 phenotype is characterized by having greater belowground biomass allocation (RMF),
419 less photosynthetic but more water-use efficient leaves (greater LDMC and C:N ratio,
420 lower SLA) and thicker and more resistant roots (lower SRL) (Fig. 1 and Fig. S5).
421 These traits are expected to be beneficial when resources are scarce, since they are
422 associated with longer lifespan and enhance water use efficiency of the plant under
423 water stress (Díaz et al., 2016). Thus, the plastic response towards a conservative
424 phenotype could improve *T. brevicorniculatum* ability to cope with water deficit. In this
425 case, AM symbiosis seems to induce direct plant trait plasticity, and not indirectly by a
426 lack of reaction to water deficit. Accordingly, the host plant with more root length
427 colonized by AM fungal (%RLC), had thinner roots, and higher SLA (Fig. S5a).

428 During the adult stage, the direction of plasticity changed compared to the
429 response at the juvenile stage. In response to reduced water availability and/or absence
430 of AM fungi plants shifted towards more resource-acquisitive phenotype. The plants
431 decreased LDMC and C:N ratio and increased their SRL and percentage of fine roots,
432 reflecting an adaptive phenotypic plasticity that improved resource uptake and
433 compensated for the lack of AM symbiosis and the involvement of extraradical
434 mycelium in plant resource uptake (Fusconi, 2014; Goh et al., 2013; Pozo et al., 2015).
435 At this stage, the plasticity triggered by AM symbiosis seems to be indirect and caused
436 by a lack of physiological reaction of the plant to water deficit (i.e., it induced the same
437 phenotypic changes as the water control condition), because AM symbiosis have
438 increased plant tolerance against stress. This different responses depending on

developmental stage could explain why previous studies found variable effects of mycorrhizal symbiosis on plant phenotype (D. Johnson, Martin, Cairney, & Anderson, 2012; Nuortila et al., 2004). Nevertheless, at both stages we found strong positive correlation between root length colonized by AM fungal (%RLC) and SRL and strong negative one between %RLC and root diameter (Fig. S5b).

Transgenerational effects on offspring performance and phenotype

Several studies had already shown that offspring from mycorrhizal parents can have greater fitness, reflected in higher biomass, survival, growth rate, and seed production (Heppell et al., 1998; Koide, 2010; Varga et al., 2013). We partially confirmed this in this study. We found that offspring of mycorrhizal parents had higher leaf P content than those of non-mycorrhizal parents (Fig. 1d), suggesting that mycorrhizal symbiosis, besides directly providing soil nutrients to host plants, could also increase offspring's nutrient uptake via transgenerational effects. However, in terms of biomass, we observed that indeed having non-mycorrhizal parents was beneficial (Fig. 1a). This contrasting result in biomass was probably due to the fact that, unlike previous evidence, we experimentally after controlling the effect of seed mass and stoichiometry (Dechaine et al., 2015; Germain et al., 2019; Herman & Sultan, 2011; but see Varga & Kytöviita, 2010). Also it could have further allowed us to detected underlying transgenerational effects probably controlled by epigenetic or hormonal mechanisms (Herman & Sultan, 2011; Rottstock, Kummer, Fischer, & Joshi, 2017; Varga & Soulsbury, 2017). One way to confirm whether these effects were only epigenetically controlled would be to modify the epigenetic signature of the plants via application of a demethylation agent (Puy, de Bello, et al., 2020; Puy et al., 2018). However, it should be first tested whether the demethylation application does not harm the AM fungal community.

Moreover, we found transgenerational effects in offspring traits linked with the resource use and exploitation strategy of the plant (Díaz et al., 2016), with transgenerational plasticity of the offspring being concordant with the within-generational response to the treatments. Plant traits shifted towards a “stress-coping phenotype under water deficit and absence of AM symbiosis during their life cycle (i.e., more conservative phenotype: increased the RMF and decrease SRL). Additionally,

471 offspring individuals became even more conservative when their parents were under
472 those stressful conditions (i.e., water deficit and absence of AM symbiosis; Fig. 1).
473 Thus, the transgenerational effects reinforced trait plasticity when individuals were
474 grown in the same conditions of their parents. This suggests a “stress memory” effect
475 that could improve the ability of plants to cope with predictable environment across
476 generations. The higher biomass found in offspring of non-mycorrhizal parents could
477 partly be a consequence of this adaptive heritable phenotypic plasticity, that enhance
478 their water use efficiency despite the environment they grow on.

479 Even though both abiotic and biotic parental environments seemed to trigger
480 transgenerational effects, we found that mycorrhizal fungal inoculation affected plant
481 traits more than water availability (Fig. 1 and Table S2). Since *T. brevicorniculatum* has
482 a wide ecological niche – it grows well under different water availability conditions
483 (Luo & Cardina, 2012) – it is likely that water is a less crucial stressor for this species,
484 and likewise that the transgenerational effects due to water availability could have been
485 not evolutionary nor ecological strongly constraining (Rendina González, Preite,
486 Verhoeven, & Latzel, 2018). Also, it is important to emphasize that we found that
487 transgenerational effects on phenotype were expressed early on the ontogeny (Fig. 1)
488 and faded away over offspring life development (Fig. 2). This result reinforces the idea
489 of transgenerational effects as an important factor promoting adaptation to repeated
490 ecological conditions, especially during juvenile stages and establishment of
491 communities (Dechaine et al., 2015; Latzel et al., 2014). Also, even though
492 transgenerational effects are likely to fade away with time, the effects associated to
493 differences in the seeds might fade away even faster (Latzel et al., 2010) as happened in
494 our case (Table S2 and S3).

495

496 *Within- and transgenerational effect on AM fungal colonization*

497 As expected, the environmental factors experienced by offspring during their life cycle
498 (Off W) affected the offspring AM fungal colonization. Contrary to our expectation
499 (Martínez-García et al., 2012; Pozo et al., 2015), water deficiency did not stimulate root
500 AM fungal colonization (since %RLC decreased). However, the proportion of
501 arbuscules:vesicles increased in offspring with water deficit, suggesting that water

502 deficiency can stimulate AM fungal activity, since the arbuscules are the main
503 structures where resource exchange takes place.

504 Moreover, we found that AM fungal colonization of the offspring could be
505 influenced by the conditions experienced by the host plant parental generation. To our
506 knowledge, this is the first study that shows transgenerational effects on AM fungal
507 colonization (but see De Long et al., 2019 for differences in AMF structures).
508 Specifically, we observed that only under water deficit conditions, the offspring of
509 parents that experienced water scarcity had lower AM fungal colonization. Although
510 this appears to contradict our initial hypothesis that resource scarcity (i.e., water deficit
511 and NM parental treatments) would stimulate AM symbiosis of the offspring, we must
512 note that offspring from water deficit and NM parents compensated by having increased
513 root biomass. This means that this offspring had in total more roots colonized and
514 greater number of arbuscules per individual than offspring from parents under ambient
515 water conditions (see the calculations made by relativizing to the total root biomass of
516 the plant – i.e., root biomass x %RLC – Fig. S6). Thus, we conclude that the result in
517 general still supports the notion that transgenerational effects modify offspring towards
518 the “stress-coping phenotype” stimulating the establishment and activity of the AM
519 symbiosis.

520 As also found for plant traits, the AM fungal colonization of the offspring was
521 influenced by the parental conditions still during adult stage. This suggests that
522 transgenerational effects could influence plant–AM fungi relationship and persist
523 further than just the establishment and early stages of the symbiosis. At the adult stage,
524 %RLC was affected by the parental mycorrhizal status, so that offspring from
525 mycorrhizal parents had higher %RLC under water deficit. These results suggest that
526 mycorrhizal symbiosis could be promoted in the offspring when the parental generation
527 had experienced mycorrhizal symbiosis.

528

529 **Conclusions**

530 We found that mycorrhizal symbiosis, alone and in combination with water availability,
531 triggered phenotypic changes within and across generations on plant performance and
532 AM fungal colonization. Water deficits and absence of AM fungi triggered concordant
533 plant phenotypic plasticity, towards a stress-coping phenotype, both within- and across

generations. This reflects an adaptive epigenetic mechanism that promotes rapid adaptation, and probably improves the ability of the species to cope with water deficit. In a context where the importance of individual and intraspecific variation of mycorrhizal plants and fungi in ecosystems is increasingly acknowledged (D. Johnson et al., 2012), our study provides evidence of a mechanism of variation that has been neglected so far: transgenerational plasticity. These plastic changes confer competitive advantages to the next generation. Importantly, we show that transgenerational effects remained significant even after controlling by the differences in seed provisioning and nutritional quality stocked by the mother, pointing to other mechanisms such as heritable epigenetic mechanisms as potential mediators and transmitters of these effects across generations.

545

546 **Acknowledgments**

We thank M. Applová, T. Jairus and N. G. Medina for technical assistance and N. Plowman for English revision. This research was financially supported by the Czech Science Foundation grant GACR (Grant No. GA17-11281S) and the European Union through the European Regional Development Fund. IH, MÖ, CPC and MM received support by grants from the Estonian Research Council (PSG293, PUT1170) and by the European Regional Development Fund (Centre of Excellence EcolChange).

553

554 **Author contribution**

JP, CPC, IH, MÖ, FdB and MM designed the research; JP performed the experiments; JP and CPC analysed the data; JP wrote the main manuscript. All authors contributed substantially to revisions and gave final approval for publication.

558

559 **Data availability**

The data that support the findings of this study will be available on Figshare repository with acceptance.

562

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753

754 **Supporting information**

755 **Fig. S1:** N and C content of the substrate (AMF and NM).

756 **Fig. S2:** Schematic representation of the design of the offspring experiment.

757 **Fig. S3:** Effect of the treatments on parental generation.

758 **Fig. S4:** Effect of the offspring and parental treatments on frequency of AM structures
759 of the juvenile and adult offspring.

760 **Fig. S5:** Correlation between pairs of plant traits and AM fungal colonization measured
761 in juvenile and adult offspring plants.

762 **Fig. S6:** Relative effect of the offspring and parental treatments on total root AM fungal
763 colonization and percentage of arbuscules on juvenile offspring.

764 **Table S1:** Summary of the linear mixed-effect model for main and interaction effects of
765 the treatments in the parental generation.

766 **Table S2:** Summary of the linear mixed-effect model for main and interaction effects of
767 offspring and parental treatments on juvenile offspring.

768 **Table S3:** Summary of the linear mixed-effect model for main and interaction effects of
769 offspring and parental treatments on adult offspring.

770 **Figures:**

771

772 **Figure 1:** Effect of the offspring and parental treatments on plant phenotype
773 characteristics of the juvenile offspring. a) total plant biomass, b) root mass factor, c)
774 leaf dry matter content, d) leaf P content, e) leaf C:N ratio, f) specific root length and g)
775 fine roots percentage. The significant factors of each model with the directionality of

776 each effect are shown in the boxes. The factors corresponded to the offspring
 777 conditions: mycorrhizal inoculation treatment, Off. M; and water availability treatment
 778 Off. W; and the parental conditions (also highlighted in bold face): mycorrhizal
 779 inoculation treatment, Par. M; and water availability treatment, Par. W. Colour coding
 780 indicates the parental treatments: red - offspring of water-deficited parents, blue -
 781 offspring of parents that experienced water control conditions; intense colour - offspring
 782 of mycorrhizal parents, light colour - offspring of non-mycorrhizal parents. The bottom
 783 and top of the boxes are the 25th and 75th percentiles respectively, the centred band is
 784 the median and the whiskers represent 1.5 times the length of the box further from the
 785 box limits or the maximum or minimum observation in the absence of outliers.

Figure 2: Effect of the offspring and parental treatments on plant phenotype characteristics of the adult offspring. a) total plant biomass, b) root mass factor, c) leaf dry matter content, d) leaf P content, e) leaf C:N ratio, f) specific root length and g) fine roots percentage. The significant factors of each model with the directionality of each effect are shown in the boxes. The factors corresponded to the offspring conditions: mycorrhizal fungal inoculation treatment, Off. M; and water availability treatment Off.

W; and the parental conditions (also highlighted in bold face): mycorrhizal inoculation treatment, Par. M; and water availability treatment, Par. W. Colour coding indicates the parental treatments: red - offspring of water-deficited parents, blue - offspring of parents that experienced water control conditions; intense colour - offspring of mycorrhizal parents, light colour - offspring of non-mycorrhizal parents.

Figure 3: Effect of the offspring and parental treatments on AM fungal root colonisation in juvenile stage (upper row) and adult stage (lower row): a) and c) percentage of root length colonized by AM fungi; b) and d) arbuscule:vesicle ratio. The significant factors of each model with the directionality of each effect are shown in the boxes. The factors corresponded to the offspring conditions: mycorrhizal inoculation treatment, Off. M; and water availability treatment Off. W; and the parental conditions (also highlighted in bold face): mycorrhizal inoculation treatment, Par. M; and water availability treatment, Par. W. Colour coding indicates the parental treatments: red - offspring of water-deficited parents, blue - offspring of parents that experienced water control conditions; intense colour - offspring of mycorrhizal parents, light colour - offspring of non-mycorrhizal parents. The nonmycorrhizal offspring treatment plants were not colonized by AM fungi.