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Influence of arbuscular mycorrhiza and cadmium on the polyamine contents of Ri T-DNA transformed *Daucus carota* L. root cultures

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ABSTRACT Influence of arbuscular mycorrhizal (AM) colonisation on free polyamine (PA) contents and ratios was investigated in vitro by Ri T-DNA transformed root cultures (*Daucus carota* L. and *Glomus intraradices* Schenck & Smith) under Cd-treatment. Roots were soaked in Cd(NO₃)₂ solutions of 3x10⁻⁶ M and 5x10⁻⁵ M concentrations for 6 hours. Roots were strongly colonised and their Cd-content increased in case of the higher Cd-treatment only. In contrast to earlier results, Cd treatment reduced putrescine and spermidine contents of the non-mycorrhizal (NM) *Daucus carota* roots, whereas that of mycorrhizal (M) roots did not change. Spermine content showed a slight increase in all cases. NM roots always had higher PA ratios. AM symbiosis may have established a more equalised environment for the roots and so decreased the volume of physiological responses induced by stress conditions, which are normally reflected by strong changes in polyamine contents.

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KEY WORDS

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spermidine

Arbuscular mycorrhiza (AM) is the most ancient and widespread form of the mycorrhiza symbiosis. AM fungi normally stimulate phosphorus uptake, growth and photosynthesis of host plants; in return, host plants provide carbohydrates for their fungal partners (Smith and Read 1997).

Heavy metal stress resistance of the plant partners is often improved by colonisation, however, a general mechanism still has not been established. The uptake of heavy metals by AM colonised plants may be higher in high heavy metal containing soils (Gildon and Tinker 1983, Guo et al. 1996), but in other cases, AM colonisation reduced the heavy metal content of plant tissues (Schüepp et al. 1987; Díaz et al. 1996). Tonin and co-workers (2001) showed the accumulation of the toxic metal in non-toxic forms in the root. Due to the greater absorbing surface, the uptake of Cu, Zn and Cd were increased by the mycorrhiza from polluted soil, but the volume of the translocation to the shoot was reduced (Loth and Höfner 1995; Joner and Leyval 1997). According to Schüepp et al. (1987), under high soil heavy metal content, Zn and Cd uptake decreased in AM plants. However, in addition to the reduction of the Cd uptake, the absorption of the Zn was stimulated when heavy metal concentrations in soil were low. The effect of the AM is strongly dependent on the type and concentration of the certain heavy metal, the pH of the environment/medium and the growth conditions. A general mechanism cannot be defined even under the same

conditions, since different fungal strains often have a mechanism specific to them (Weissenhorn et al. 1995).

The accumulation of the Cd in the fungal structures with large heavy metal binding capacity may act as a biological 'barrier' system (Joner and Leyval 2001). Turnau and co-workers (1993) showed in *Pteridium aquilinum* roots, that heavy metals (Cd and Ti) were sequestered in the vacuoles intercellularly and in the extramatrical hyphae.

It is still unclear to what extent the inherent stress resistance mechanisms of the host plants are influenced by the presence of mycorrhiza, which may partly explain the great diversity in the heavy metal stress responses of AM plants according to Rivera-Becerril et al. (2002). The overall improved heavy metal (Cd) resistance of AM plants was called the 'buffer-effect' of the mycorrhiza (Rivera-Becerril et al. 2002), but detailed characterisation of this effect is still to be made.

Change in the hormonal balance (including polyamines) is a frequent response of plant metabolism to the mycorrhizal colonisation influencing many physiological aspects including stress resistance (Smith and Read 1997). Furthermore, several environmental challenges like mineral nutrient deficiencies or atmospheric pollutants (e.g. Cd) have profound effects on plant polyamine (PA) metabolism (Bouchereau et al. 1999). Putrescine (Put) seems to be a stress signal molecule and its concentration is usually increased under stress conditions. Our knowledge is very limited about the role of PAs in plant-microbe symbioses (Walters 2000), but they may take part in the molecular

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signalling events between the symbiotic partners (El Ghachtouli et al. 1995). Their concentrations and ratio may reflect the demand for the AM symbiosis in P deficiency stress conditions (Parádi et al. 2003). Since they have a basic role in stress physiology, PAs may also have a role in the improved heavy metal stress resistance of AM plants.

Materials and Methods

Ri T-DNA transformed carrot (*Daucus carota L.*) roots were provided by Dr. Guillaume Bécard (Lab. de Mycol. Pole de Biotech. Veget., Auzeville, Castanet Tolosan, France). Mycorrhizal root cultures were colonised by *Glomus intraradices* Schenck & Smith. Roots were cultivated on a minimal (M) medium described by Bécard and Fortin (1988) solidified with 0.3% Phytigel® (Sigma-Aldrich, St. Louis, MO, USA). After subculturing, roots were grown for six weeks in the dark at room temperature.

During treatments, roots with the agar discs were transferred to 1 L beakers containing 100 ml $1/4$ -strength Hoagland solution (Fodor et al. 1998). For Cd treatment, $\text{Cd}(\text{NO}_3)_2$ in 3×10^{-6} M (Cd-1) and 5×10^{-5} M (Cd-2) concentrations were used. One of the controls was measured immediately after opening the Petri dish (Co-1), while the other control was soaked for 6 hours on a shaker (120 rpm) with the Cd-treated roots (Co-2). After treatment, root fragments were thoroughly cleaned of agar remnants and randomly homogenised.

Mycorrhizal colonisation was quantified according to Phillips and Hayman (1970) and Trouvelot et al. (1986). Cd contents of roots were measured by an JY238 Ultrace ICP spectrometer (Jobin Yvon/Spex Division, Longjumeau, Cedex, France) in the ICP laboratory of the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest, Hungary.

Quantities of free PAs were determined by thin-layer chromatography (Rácz et al. 1996) and by a Jobin Yvon-Spex Fluoromax-2 spectrofluorometer (Instruments S.A., Jobin Yvon/Spex Division, Longjumeau, Cedex, France). Put content compared to the total of spermidine (Spd) and spermine (Spm) contents (Put/(Spd+Spm) ratio) was calculated.

SPSS 7.5 was used to perform two-way ANOVA on the effects and interaction of treatments. Means were compared between treatments by the Student's *t*-test.

Results

Colonisation

The colonisation parameters of the mycorrhizal (M) roots were relatively high and had similar values. The average colonisation frequency (F%), the intensity of the colonisation (M%) and the arbuscule content of the roots (a%) were 92%, 75% and 42%, respectively. As expected, colonisation was not found in the non-mycorrhizal (NM) root cultures.

Cd uptake

The amount of Cd entering the roots in Cd-1 treatment did not differ from the control value. However, despite the lack of deviation (there was only one parallel measurement), it can still be established that heavy metal content of roots was much greater in case of Cd-2 treatment (Table 1).

Polyamine content

The Cd treatments and the presence of mycorrhiza had significant effects on the contents of PAs in all cases (Fig. 1, Table 2). The Put content of the NM roots decreased under both Cd treatments compared to the control values, but the decline was not affected by the amount of Cd used (Fig. 1). Typically, Put contents of the NM roots were always higher than those of the M roots, and the effect of the mycorrhiza was highly significant (Table 2). The Put content of the M roots did not change substantially.

The Spd content also decreased in the NM roots when treated with Cd, irrespectively of the amount of the Cd in the media. The soaking must have played a role as well as the presence of the Cd, in the decreasing of the levels of Spd. Examining the Co-2 treatment, the effects of soaking and Cd treatments can be separated (Fig. 1). The Spd contents of the M roots changed slightly under the different treatments. They were lower without and higher with Cd treatment than those of the NM roots. This is confirmed by the fact that the interaction of mycorrhiza and Cd treatments was highly significant as regards Spd content (Table 2). It can be concluded, that the extent of changes in the Spd contents caused by the heavy metal treatment are dependent on the presence of mycorrhiza.

In both the M and the NM roots, a slight increase was observed in the Spm levels after Cd application (Fig. 1), which proved to be significant (Table 2).

Similarly to the differences in the Put content, the effect of the mycorrhiza on the Put/(Spd+Spm) ratios was also significant (Table 2.), in mycorrhizal roots these were lower in all cases (Fig. 1).

Discussion

Colonization and Cd uptake

According to di Toppi and Gabbrielli (1999), soils containing Cd concentrations above 1 μM can be considered as being strongly polluted. In this study, the applied treatments of 3 and 50 μM Cd are within the lower range of the strongly polluted category.

The potential heavy metal binding capacity of the media might have influenced the amount of Cd entering the root tissues, which may explain why the Cd content of roots under the lower Cd treatment was very similar to that of the control (Table 1). It can also be hypothesised based on previous results (Turnau et al. 1993) that Cd might have also been

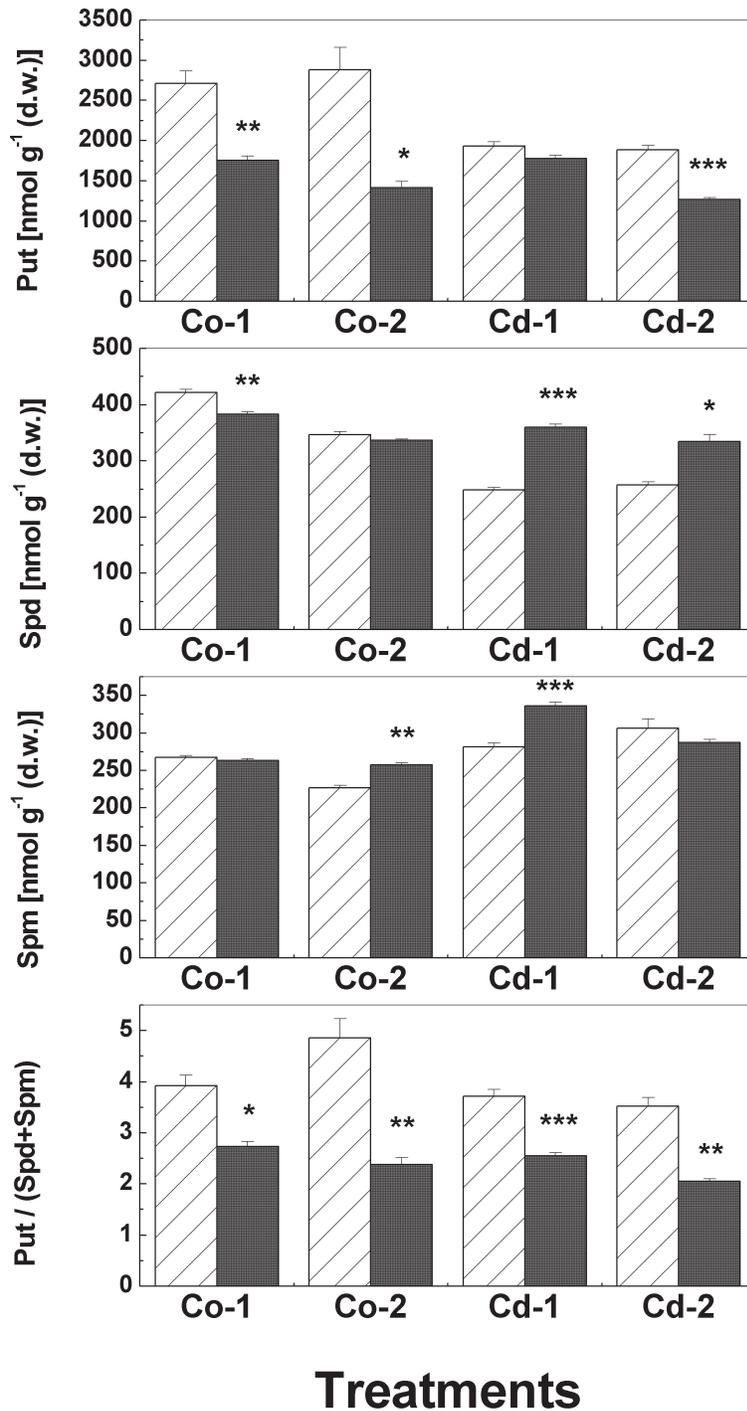


Figure 1. Average putrescine (Put), spermidine (Spd) and spermine (Spm) contents and polyamine ratios (Put/(Spd+Spm)) in mycorrhizal (M) and non-mycorrhizal (NM) Ri T-DNA transformed *Daucus carota* root cultures regarding to dry weight. SE is shown by vertical bars. Statistically significant differences between M (closed columns) and NM (hatched columns) plants under the same treatments are indicated as: *-P≤0,05, **-P≤0,01, ***-P≤0,001.

bound by the external hyphae of mycorrhizal fungi leading to lower Cd content of M roots in case of Cd-1 treatment.

The higher dose of Cd caused greater (1.5 times higher) accumulation in the M roots compared to the NM (Table 1). There is no information about the precise localisation of the Cd in the tissue, but it was postulated before (Joner and Leyval 1997) that substantial amount of Cd can be immobilised in the intra- or intercellular hyphae or structures of the mycorrhizal fungal partner. The toxic effect of Cd on the M root tissues could have been reduced by this immobilisation, in spite of the higher overall metal content. The accumulation of the Cd in a large heavy metal binding capacity fungal structure may act as a 'biological barrier' (Joner and Leyval 2001). The role of mycorrhizal fungi in adsorbing Cd can also be supported by the relatively high colonisation intensity in the present study.

Polyamine content

El Ghachtouli et al. (1995) were the first to show that PAs may have a role in the AM symbiosis, when colonisation frequency in peas was increased by applying PAs exogenously. El Ghachtouli et al. (1996) later proved that root growth and AM colonisation could be reduced by the application of PA biosynthesis inhibitors. This effect was reversed by the addition of exogenous Put. However, in the same experiment, no difference was found in the PA concentrations between mycorrhizal and control plants. Nevertheless, Goicoechea et al. (1998) found higher Spd and Spm concentrations in mycorrhizal alfalfa plants under water stress conditions.

It is well known that K deficiency has a considerable effect on the level of Put (Richards and Coleman 1970, Savonen and Sarjala 1998), and there is data on the possible role of the P deficiency (Parádi et al. 2003). In the present study, the roots were grown on a minimal media for the optimal growth and function of the mycorrhiza. This media contained low amounts of K and P (KH_2PO_4 : 4.8 mg/l). Therefore, the higher Put levels and PA ratios observed in the NM roots (Fig. 1) can be also explained by the effect of the low K and P supply.

Table 1. Total Cd content of mycorrhizal (M) and non-mycorrhizal (NM) Ri T-DNA transformed *Daucus carota* root cultures.

Treatment	Cd concentration (mg/kg d.w.)
NM Co-1	0,33
M Co-1	1,00
NM Co-2	0,32
M Co-2	0,28
NM Cd-1	0,988
M Cd-1	0,492
NM Cd-2	106
M Cd-2	164

Treatments: without soaking and Cd (Co-1), soaking without Cd (Co-2), soaking in $\text{Cd}(\text{NO}_3)_2$ solution with 3×10^{-6} M and 5×10^{-5} M (Cd-1 and Cd-2) concentrations.

The decline of the amount of Put under Cd treatment in NM roots (Fig. 1) is in contrast to some previous results. Put levels increased at the concentrations of 10 and 25mM Cd in oat (*Avena sativa*) and bean (*Phaseolus vulgaris*) leaves (where the changes may be different from the roots; Weinstein et al. 1986), and at the concentration of 50 mM Cd in barley (*Hordeum vulgare*) leaves and roots (Soós 2000). However, no alteration in Put levels was detected after Cd treatment in potato leaves (Stroinski and Szczotka 1990), but the level of Spd and Spm were considerably increased. The present study was carried out on a different plant species, under unique growing conditions using root cultures, and the samples were taken at the sixth hour of the treatment, unlike to the 1-5 days incubation times used in the studies mentioned above (Weinstein et al. 1986; Stroinski and Szczotka 1990; Soós 2000).

Soós (2000) observed differences between the changes of PA levels in the root and leaves under Cd stress conditions, as it has also been revealed in the case of P deficiency (Parádi et al. 2003). Leskó and co-workers (2002) studied Cd stress in wheat (*Triticum aestivum*). According to their results, no changes in the amount of Put was observed under low Cd stress (0.1 μM Cd), but the level of Spd rose sharply in the leaves and similar to the results of the present experiment, decreased in the root (Fig. 1). Whereas when Cd was applied in high concentration (1 mM), Put level showed an increase in the root, while the amount of Spd rose only slightly (Leskó et al. 2002).

A decline was observed in the amount of the Spd showing a similar trend to the Put levels in the NM roots when treated with Cd in this study (Fig. 1). The rates of the changes in the levels of Put and Spd in the NM roots were independent from the concentration of the Cd applied and the Cd contents of the tissues (Table 1), because their amounts were the same both in the cases of Cd-1 or Cd-2 treatments (Fig. 1). Therefore, it is postulated, that like the effect of the P deficiency (Parádi et al. 2003), Cd induced changes in PA contents might be observed above a certain level of the heavy metal and they were not connected strictly to the actual external Cd concentration under the experimental conditions of this work.

According to Weinstein and co-workers (1986), the level of Spm doubled after 24 hours Cd incubation in oat and bean leaves. Further investigations may reveal whether the moderate but steady increase of Spm levels (Fig. 1) could improve under additional incubation.

The increase in the amounts of Spd and Spm probably indicates stress tolerance, while Put causes membrane depolarization and K^+ release. Put levels normally go up under many stress conditions in sensitive plants, whilst Spd and Spm accumulate in the tolerant plants (Rácz et al. 1996; Bouchereau et al. 1999). PAs participate in the quenching of free radicals, the delay of senescence and the inhibition of

Table 2. Significance (ANOVA) of effects of mycorrhizal colonization and soaking/Cd treatment on polyamine contents and ratio and their interaction in *Daucus carota* root cultures.

	Put content	Spd content	Spm content	PA ratio
Mycorrhiza	***	***	ns	***
Treatment	*	***	***	ns
Interaction	ns	***	**	ns

Levels of significance: ns-non significant, *-P≤0,05, **-P≤0,01, ***-P≤0,001.

lipid peroxidation. They retard the increase in the superoxide levels proportional to their chain length (Bouchereau et al. 1999). Based on the role PAs play and the degree of their accumulation under different stress conditions, the Put/(Spd+Spm) ratio was defined as a suitable stress indicator parameter (Minocha et al. 1997). The general difference observed between PA ratios of the NM and M roots (Fig. 1) emphasises the marked effect of the presence of mycorrhiza on the PA metabolism and indicates a lower degree of stress in the colonised roots.

To conclude, no considerable changes in the Put and Spd contents of the roots of mycorrhizal *Daucus carota* were observed after Cd treatment, they remained similar to the control values (Fig. 1). The presence of mycorrhiza seemed to have a kind of 'equalising' effect under the Cd stress against the changes in PA contents widely described in various stress conditions (Bouchereau et al. 1999). By supplying nutrients and adsorbing heavy metals, the mycorrhiza can provide a balanced environment for the roots. This 'buffer-effect' (Rivera-Becerril et al. 2002) may alleviate some of the physiological alterations induced by stress conditions. The significant interaction between the Cd treatments and colonisation supports this theory (Table 1), since the effect of Cd on the roots was reduced in the presence of mycorrhiza. Nevertheless, mycorrhiza could have also influenced the hormonal balances of roots growing on the minimal media by supplying P and other nutrients.

Finally, it is presumed, that subsequent upon the direct hormonal or the indirect nutrient supply and/or morphological effects of the mycorrhizal colonisation, the roots can get into a different state, which is reflected by the Put and Spd contents and the ratio of PAs. The observed differences in the PA contents of the *Daucus carota* root cultures may allude to an increase in the tolerance to Cd stress in M roots. Further investigation is needed to elucidate the potential ways of the improved stress tolerance of AM colonised roots.

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