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The effect of inoculation with arbuscular mycorrhizal fungus *Rhizophagus irregularis* on cytokinin content in a highly mycotrophic *Medicago lupulina* line under low phosphorus level in the soil

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ABSTRACT

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The study is focused on the elucidation of the role of cytokinins (CKs, zeatin and zeatin riboside) in the development of effective arbuscular mycorrhiza (AM) symbiosis with *Medicago lupulina*. An important mechanism involved in the regulation of host plant growth is supposed to be linked to the modulation of plant hormone balance. The data obtained revealed the formation of an effective AM-symbiosis (*M. lupulina* + *Rhizophagus irregularis*) under phosphorus-deficiency. At the shooting stage (35th day after sowing), it is characterized by a decrease in the root:shoot ratio, the lowering in arbuscules and vesicle abundances, but an increase in the intensity of mycelium development. Mycorrhized plants differed from the control ones by higher CK levels in both roots and leaves. Zeatin and zeatin riboside concentration exhibited uneven alterations over time. A role of mycelium in the modulation of CK balance has been discussed.

Keywords: Fabaceae; phytohormone; rhizosphere; macronutrient; arbuscular mycorrhizae

Arbuscular mycorrhizae (AM) is a mutualistic nonspecific symbiosis formed by most land plant families (92%) with fungi represented by the Glomeromycota phylum. This type of symbiosis is

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based on the reciprocal exchange of soil nutrients, primarily phosphate and carbohydrates, between fungi and the host plant. AM-fungi might consume up to 20% of host plant photosynthates but simultaneously enhance plant growth (Smith and Read 2008). This intensification commonly depends on the alteration in plant hormone balance, which has become an object of special interest in recent years (Fusconi 2014, Boivin et al. 2016, Yurkov et al. 2017).

Cytokinins (CKs) are among essential regulators of root growth. However, published data are highly controversial (Fusconi 2014, Boivin et al. 2016). Both exogenous and endogenous CKs, as well as defects in CK synthesis and sensing, modulate lateral root formation in *Arabidopsis* and therefore AM formation (Fukaki and Tasaka 2009, Bielach et al. 2012). AM plants were shown to accumulate more CKs in roots and shoots in comparison to non-mycorrhized plants. CK elevation was more pronounced at later stages of mycorrhization (Danneberg et al. 1993). A strong correlation between increased CK levels, intensified photosynthesis and enhanced growth of AM plants allows for the establishment of the hypothesis that cytokinin is a part of the positive AM effect on the host plant (Drüge and Schonbeck 1992). But such a positive effect has not been identified in all cases. For example, exogenous CKs are able to reduce symbiosis formation (Schmidt et al. 2017). The endogenous CK levels might be decreased in AM roots under high levels of available inorganic phosphorus (P) in soil (Torelli et al. 2000). They may also be associated with unequal changes in CK content in root and shoot (Danneberg et al. 1993, Cosme et al. 2016). Thus, additional investigation of AM-induced CKs is required.

In the present study, AM-induced alteration in CK levels was tested in a *Medicago lupulina* MlS-1 line that was characterized by significant responsiveness to *Rhizophagus irregularis* inoculation at different stages of host plant development and under conditions of low P level in the soil. The alteration in CK level was compared with the dynamics of mycorrhizal effectiveness and modulation of mycorrhiza structures.

MATERIAL AND METHODS

Plant and fungal materials. Black medick (*Medicago lupulina* L. var. *vulgaris* Koch, annual) is known to establish symbiosis with the strain RCAM00320 of AM fungus *Rhizophagus irregularis* (previously known as *Glomus intraradices* Shenck&Smith; strain CIAM8 from the collection of the All-Russian Research Institute for Agricultural Microbiology; Yurkov et al. 2010). The selected MlS-1 line from cultivar-population VIK32 was used in the present study. Plants of this line show signs of dwarfism in the absence of inoculation with AM fungus and at low P levels in the soil.

Analyses were performed on: 1st day after sowing (DAS) – seedling stage; 14th DAS – the stage of the first true leaf; 21st DAS – the stage of the second leaf development; 35th DAS – the shooting stage; 50th DAS – the initial stage of flowering.

Microvegetation method. Microvegetation method provides optimal conditions for AM development and allows avoidance of spontaneous infection by rhizobia and other symbiotic microorganisms. The substrate for growing (air-dry soil-sand mixture in 2:1) was autoclaved twice. Plants were grown in a UV-sterilized light chamber with inactive ventilation under standard conditions (luminous flux at ~1500–1700 lm, a 16h/8h day/night cycle, temperature of ~24–26°C, ambient air humidity ~60–65%, plant watering up to 0.6 of saturated water content, Yurkov et al. 2010). Four seedlings were planted per 1 pot, filled with soil-sand mixture (210 g). Agrochemical soil parameters were described earlier (Yurkov et al. 2010), P level was 17 mg/kg.

Light microscopy. Light microscopy was used to assess the main AM quantitative and qualitative characteristics (Trouvelot et al. 1986). AM structures such as arbuscules, vesicles and mycelium were determined at flattened root samples, stained, macerated, and cut in 1 cm pieces (in length) of host plant roots (Phillips and Hayman 1970). Mycorrhiza software (developed by A.P. Yurkov) was used to calculate the values of mycorrhization in a number of the visual optical fields (1.69 mm) in order to ensure a corresponding level of accuracy (significance level for classes of mycorrhizal density is 5%).

Cytokinin determination. Concentrations of CKs (*trans*-zeatin (Z) and its riboside (ZR)) in the roots and shoots were detected. Samples were homogenized and CKs were extracted in 80% ethanol. The extract was separated from plant debris by centrifugation and the ethanol evaporated. Aliquots of the rest aqueous residues were loaded on a C18 cartridge (500 mg, Varian, Middelburg,

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the Netherlands), washed with distilled water. CKs were eluted with 70% ethanol and the eluate evaporated to dryness and dissolved in a minimum of 80% ethanol. Thin-layer chromatography (TLC) was used for Z and ZR separation (Vysotskaya et al. 2009). CKs were determined by an enzyme immunoassay method as described earlier (Kudoyarova et al. 2014). Anti-cytokinin antibodies with high immunoreactivity towards *trans*-zeatin and its riboside showed an inherently low cross-reactivity to other CKs including *cis*-zeatin and its derivatives.

Minimal biological repetition was 12 plants per each variant. The significance of differences between the variants was assessed by the *t*-Student criterion at the significance level $P \leq 0.95$.

RESULTS AND DISCUSSIONS

Development of black medick passed through several physiologically important stages: 1st and 2nd true leaf, shooting and the beginning of flowering, all used as time points for further analysis. The present study demonstrated that AM significantly affected plant development. As one criterion, the ratio of root fresh weight (RFW) to shoot fresh weight (SFW) was established (Figure 1). In control plants (without inoculation) at all stages, RFW was higher than SFW. The ratio sharply exceeded 1.0 on the 21st day, namely the stage of 2nd true leaf. It then decreased slightly but was kept at a rather high value. This revealed the importance of the root system in P deficiency and the lack of nutrients supplied by the micro-symbiont. Early on, the root:shoot ratio was shown to be increased under P deprivation in *Nicotiana tabacum* (Fusconi 2014), and also in species such as *Arabidopsis thaliana* (López-Bucio et al. 2002), *Gossipium hirsutum* (Price et al. 1989), *Hordeum vulgare* (Drew 1975), *Triticum aestivum* (Adalsteinsson and Jensen 1989), and *Zea mays* (Mollier and Pellerin 1999). The root:shoot value in AM-plants was rather stable and significantly lower than in control plants, and at all time-points below one. The data obtained is in agreement with the role of AM as a facilitator of the host-plant root function.

The basic function of AM is supposed to be a supply of the host-plant by P. Thus the evaluation of AM-efficiency is usually tested as mycorrhizal effectiveness (ME, %) (Gai et al. 2006). ME attributed to shoot and root P content in *M. lupulina* was detected in the present investigation: shoot P content mycorrhizal effectiveness (SPME) and root P content mycorrhizal effectiveness (RPME), accordingly (Figure 2). Dynamics of AM-induced P accumulation was similar both in root and shoot. The maximum value of mycorrhizal effectiveness was reached on the 35th DAS. RPME was higher at all tested time points. Surprisingly the ratio RFW/SFW did not reveal significant changes in this period (Figure 1).

It is well known that the arbuscule is the 'organ' of mycorrhiza which is responsible for P uptake (Fitter 2006). The next step of this study was the investigation of arbuscule formation in comparison with other symbiont structures, mainly mycelium. The intensity of arbuscular accuracy had an unlinear character with the maximum on the 21st DAS (Figure 2). The increase of arbuscular number preceded the maximum of ME. Then arbuscular frequency decreased significantly. The intensive alteration might be due to the short life-time of arbuscule, which is limited to 4–5 days, and its fast degradation within only 2.5–5.5 h (Denison and Kiers 2011). Hovever, arbuscules are formed *de novo* at all stages of AM development (Yurkov et al. 2015). Thus, the host plant would actively control the number of arbuscules, for example, due to the

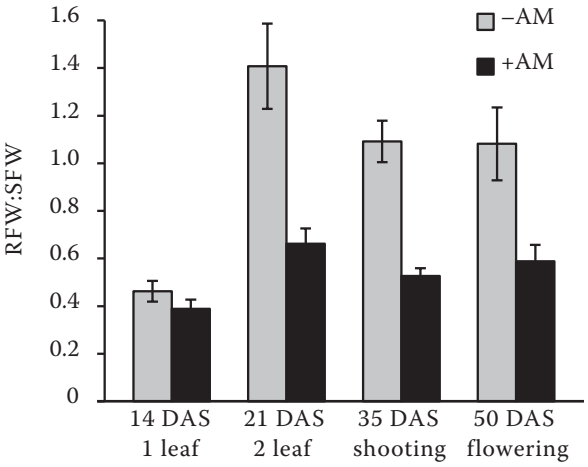

Figure 1. The ratio of root fresh weight (RFW) to shoot fresh weight (SFW) of *Medicago lupulina*. +AM (arbuscular mycorrhiza) and –AM – with and without inoculation by AM fungus, accordingly. The stages of plant development are presented at the bottom of the figure. Each value of the ratio with inoculation by AM fungus was significantly ($P \leq 0.95$) different from the control for each plant stage except the 1st true leaf stage. DAS – day after sowing

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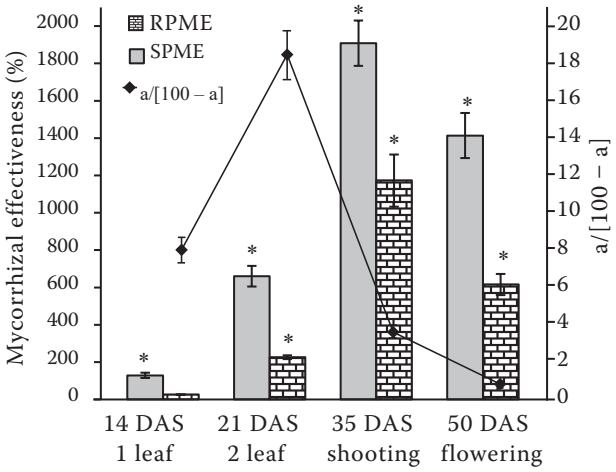

Figure 2. Mycorrhizal effectiveness (ME) (based on shoot and root phosphorus (P) content in *Medicago lupulina*) and the ratio of intensity of arbuscule development within infected areas (a, %) to the intensity of mycelium development ([100 – a], %) within infected areas in the roots. SPME (shoot P content mycorrhizal effectiveness), RPME (root P content mycorrhizal effectiveness) and ME based on shoot and root P content, is accordingly: ME = [P + AM – P – AM]/P – AM, where P_{+AM} and P_{-AM} is P content in plants with and without inoculation by arbuscular mycorrhiza (AM) fungus. Each value of ME is significant ($P \leq 0.95$) except RPME at the 1st true leaf stage; a/[100 – a] is significantly ($P \leq 0.95$) different in each stage of plant development

redistribution of carbohydrates (Schaarschmidt et al. 2007). The high level of SPME and RPME observed in this study at late stages of plant growth corresponded to the increase of mycelium. Its importance in the symbiotic exchange mechanism is still under discussion. There is some evidence that it also participates in supplying the plant with P. For example, the liquid surroundings of AM hyphae in intercellular spaces contain higher concentrations of P than those in the non-inoculated roots (Ryan et al. 2003). Moreover, the content of P is much higher in the intercellular hyphae in comparison to arbuscular trunks and branches. The active role of mycelium in plant-microbe exchange is quite well demonstrated in types of AM where the colonization is established without arbuscules (Dickson 2004).

As observed, the mycrosymbiont limited root development in comparison with the non-inoculated control, which corresponds well to earlier data (Fukaki and Tasaka 2009). There is no doubt that plant development is under the control of plant hormones. For example, CKs affect root architecture and probably act as a negative regulator of lateral root development under mycorrhization (Hodge et al. 2009, Fusconi 2014). The higher CK content in AM plants is in line with the reduction in the root:shoot biomass ratio, which occurs when colonization is established (Fusconi 2014). Larger root systems were observed in plants with low CK levels (mutants with genetic defects, encoding CK biosynthetic enzymes, transgenic *Arabidopsis* and tobacco plants with enhanced root-specific CK degradation, and plants treated with anti-CKs) (Arata et al. 2010, Kudoyarova et

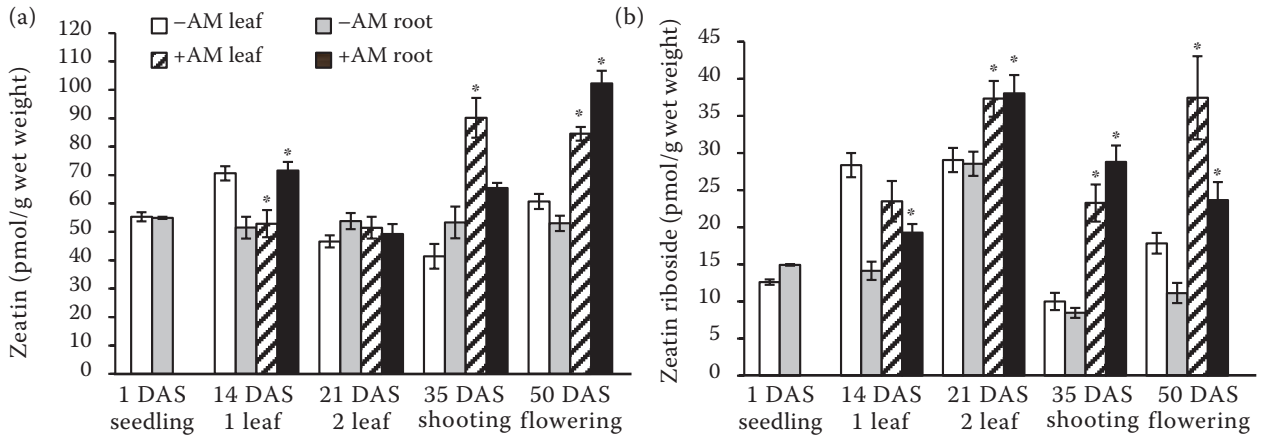

Figure 3. Concentration (pmol/g fresh weight) of zeatin (a) and zeatin riboside (b) in leaves and roots of *Medicago lupulina* non-mycorrhized and mycorrhized plants (notes are presented in Figure 1). *Value with inoculation by arbuscular mycorrhiza (AM) fungus which is significantly ($P \leq 0.95$) different from the value in control without inoculation

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al. 2015 and the references therein). A decrease in the cytokinin content under conditions of nutrient deficiency was accompanied by an increase in the root:shoot mass ratio (Vysotskaya et al. 2009).

The results of CK analysis are presented in Figure 3. *Trans*-zeatin and its riboside were determined in root and shoot of plants at different stages of development and during AM establishment. Although *cis*-zeatin is known to be present in high concentrations in plants (Gajdošová et al. 2011) and *cis*- and *trans*-forms were not separated by the TLC applied, low cross-reactivity of antibodies to the *cis*-forms of zeatin enabled preferential measurement of the *trans*-forms. *Trans*-zeatin and its riboside showed quite different dynamics. The development of control plants did not reveal intensive alterations in Z concentration. The only slight increase was detected at a very early stage of the 1st true leaf. The inoculation of plants triggered an increase of leaf Z level at later stages of the host plant development. The maximum was reached on the 35th day (shooting) and was maintained by the 50th day (flowering). In AM roots, Z level altered in a wavelike pattern. The first increase was detected on the 14th day, then it went down and was elevated again almost twice by the 50th day at the flowering stage. ZR had quite a different alteration. Control plants accumulated ZR at early stages (at 1st and 2nd true leaf). This increase was detected first in the leaves, then in roots. Later on, ZR concentrations dropped down almost up to the seedling level and was maintained up to the end of the experiment. Development of symbiosis effected ZR level in roots and at all the tested stages of host plant development apart from the 1st true leaf, however, it was very unstable.

Similarly in *Nicotiana tabacum* under P-starvation the level of ZR was always higher in AM-plants in comparison with the control (Shaul-Keinan et al. 2002); in *Linum usitatissimum* higher concentrations of ZR in leaves and roots were revealed in AM-plants even under standard P-fertilization (Drüge and Schonbeck 1992). Elevation of ZR content in mycorrhized plants was clearly demonstrated under stress conditions (Liu et al. 2016). It was suggested that AM fungi enhanced sink strength and promoted photosynthesis due to ZR. Moreover, ZR was involved in transduction of nutritional signals, and thus, slowing down of the process of senescence in target plants (Alazem and Lin 2015). The origin of ZR in mycorrhized plants is still under discussion but there is some evidence that AM fungi might synthesize CK, as in axenic mycelial cultures (Barea and Azćon-Aguilar 1982). Thus, a possible role of mycelium might be considered, but further investigation is needed to test this supposition.

In short, the effective AM-symbiosis (*M. lupulina* + *R. irregularis*) under P-deficiency was formed at the shooting stage (Figure 2) and is characterized by a decrease in root:shoot ratio (Figure 1), decrease in arbuscule abundance (Figure 2), but increase in intensity of mycelium development, as well as increase in ZR concentration (Figure 3). The following hypothesis is proposed: the intraradical intercellular mycelium is active in nutrient exchange including P-transfer from fungus to the host plant, and might affect CK balance. This phenomenon is more pronounced at later stages of AM establishment, when the representation among AM structures is turned from arbuscules to mycelium. The data presented here show the way toward possible further investigations focused on the mechanisms of CK synthesis and P supply.

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