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Effect of a Non-Mycorrhizal Endophyte Isolated from *Mentha piperita* on in Vitro Culture of *Ocimum basilicum* Cuttings

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Abstract

Fungi form different types of long-term associations with plants, from mutualistic to pathogenic. Fungal endophytes, which live within host tissues, may confer stress tolerance to the associated plants increasing plant fitness and biomass. In vitro micropropagation and large scale production of aromatic and medicinal plants may benefit from endophyte inoculation. Beside increasing plant growth, fungal endophytes may allow for a reduction of the amount of plant growth regulators in culture media and can be employed to increase the survival rates of micropropagated plants during acclimatization in soil and alleviate the transplantation shock. Previous experiments have shown that a plant growth promoter-hyaline sterile fungus (PGP-HSF) isolated from *Mentha piperita* L. increased plant growth and influenced the essential oil composition in the same species. In this paper we analysed the *SSU* rDNA *regions of PGP-HSF to determine/confirm its systematic position, which is still uncertain, and* investigated the effect of the fungus on in vitro sweet basil (*Ocimum basilicum* L.) cuttings cultured on a diluted MS medium. The Bayesian and maximum likelihood analysis of *SSU* rDNA *regions* confirmed the previously recognized collocation of PGP-HSF in the Sordariomycetes, positioning it as a sister taxon of clade *Ophiostomataceae.* After 35 days of co-culture of *O. basilicum* with the fungus, most of the growth parameters were significantly increased. Plant fresh and dry weights, number of leaves, as well as the total leaf area per plant showed an increase greater or equal to 100% in inoculated plants as compared with non-inoculated control plants. The root-to-shoot biomass ratio also increased. These results encourage us to deepen the effects of this endophyte on basil, by studying its influence on the production of essential oils and the post-in vitro culture.

Keywords: Ascomycota, fungal endophytes, Lamiaceae, morphometry, root system, sweet basil

INTRODUCTION

In natural and agro-ecosystems most herbaceous plants are symbiotically associated with mycorrhizal fungi and/or other fungal endophytes which influence the development and fitness of their hosts. Among them, arbuscular mycorrhizal (AM) fungi are obligate biotrophs of the roots of almost all herbaceous plant species and usually promote plant growth by improving plant uptake of P and other immobile nutrients (Smith and Read, 2008). Besides mycorrhizal fungi, many other fungal endophytes are involved in symbiotic interactions with plants and can be isolated from different host tissues as roots, stems and leaves (Rodriguez et al., 2009). In general, fungal endophytes play an important role in determining host resistance to fungal pathogens and pests, to optimize the absorption of nutrients and to increase the tolerance to abiotic stress such as temperature, pH, salt stress (Smith and Read, 2008). A peppermint endophyte, the ascomycetous plant growth promoter-hyaline sterile fungus (PGP-HSF), isolated from the host stem, has shown to enhance vegetative growth and to positively modify the essential oil composition in peppermint (Mucciarelli et al., 2003).

It is known that Lamiaceae species, including basil, show a high individual variability, due to genetic and biochemical heterogeneity (Shetty, 1997). Moreover seed-derived plants are not true to type, due to cross pollination. Micropropagation techniques are suitable for the rapid and large-scale propagation of medicinal and aromatic plants, and for testing the effect of beneficial fungi. AM fungi have been shown to significantly affect plant growth, density of glandular trichomes and the quality and quantity of essential oil in sweet basil (Copetta et al., 2006); nevertheless, these fungi are not always amenable to inoculate plants in vitro.

The objective of the present study was to verify the effect of PGP-HSF in sweet basil in order to assess the possible use of this fungus to improve the micropropagation of this economically important aromatic and medicinal species.

MATERIALS AND METHODS

Plugs from the young part of mature colonies of PGP-HSF grown on MS medium (Murashige and Skoog, 1962) were co-cultured with shoot cuttings of *Ocimum basilicum* L. "Italiko" (Fig. 1A) in squared Petri plates containing a 0.2× MS medium. Control plants were not inoculated. Plates were placed vertically in a growing chamber (26/22±1°C, 16/8h light/dark photoperiod, 90 $\mu mol \cdot m^{-2} sec^{-1}$). After 35 days of culture, the fresh and dry weights of shoots and roots of 10 plants per treatment were determined. Root systems and leaves were scanned; root length and leaf area were measured by image analysis. The numbers of roots of each order and of leaves were counted and the degree of root branching was calculated.

All parameters analysed were compared between inoculated and non-inoculated plants using Student's t-test. Statistical analyses were performed using R.

In phylogenetic analysis, SSU PGP-HSF sequence was compared with those available in Genbank used for previous phylogenetic studies in Sordariomycetidae. The alignment was carried out using MAFFT v 7.017 (Katoh et al., 2002) with default conditions. Phylogenies (Bayesian and Maximum-likelihood analysis; BPP = Bayesian posterior probabilities and MLB = maximum likelihood bootstrap) were inferred with MrBayes v. 3.2.4 (Huelsenbeck and Ronquist, 2001) and RAxML v.7.2.8. (Stamatakis, 2006). MEGA v.6.06 (Tamura et al., 2013) was used to estimate the best-fit models of nucleotide substitution. The GTR + G + I model was chosen for both analyses. Bayesian inference (BI) was performed with four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) run over one million generations; trees were sampled every 200 generations and the first 25% trees were discarded as "burn-in". The MP bootstrap analysis was conducted using 1000 replicates with rapid bootstrapping.

RESULTS AND DISCUSSION

After 35-d inoculation, the mycelium of the fungal endophyte PGP-HSF had grown into the MS medium in contact with sweet basil roots (Fig. 1B). By this time, sweet basil plants were healthy and their total biomass was statistically different from uninoculated plants (Fig. 1C). Plant fresh and dry weights showed an average 160% and 181% increase over the control plants (Fig. 2A,B), due to an increase of the fresh and dry weights of both roots and shoots (Fig. 2C-F).

Plant growth stimulation by the fungal endophyte occurred along with a significant increase of the average number of leaves (from 11 in control plants to 29 in treated plants) and of stem nodes per plant (+153% and +165% respectively; $p<0.01$; Fig. 2L,M).

Sweet basil micropropagation is usually achieved by axillary shoot proliferation from nodal explants (Ahuja et al., 1982; Begum et al., 2000; Shahzad and Siddiqui, 2000), shoot tip explants, leaf explants (Phippen and Simon, 2000) and axillary buds (Begum et al., 2000, 2002). A greater number of axillary buds and nodes per plants as those obtained after PGP-

HSF inoculation will thus improve the process of micropropagation of sweet basil, by shortening the time required for each multiplication cycle with an expected reduction of production costs.

Higher number of nodes and leaves resulted in a significantly larger (116%; $p<0.01$) total leaf area per plant (Fig. 2N), which was also observed in peppermint after inoculation with the same fungus (Mucciarelli et al., 2003).

With regards to the root system of sweet basil plants, PGP-HSF inoculation resulted in a significant increase of the number of roots (+129%; $p<0.01$)(Fig. 2I), and the mean length of the whole root apparatus was almost twice the control, although at $p = 0.059$ (Fig. 2H). PGP-HSF inoculation of sweet basil plants resulted in a greater root-to-shoot ratio (Fig. 2G) as a consequence of the increase of biomass, greater in roots than in shoots, as it occurs in peppermint plants (Mucciarelli et al., 2003). This parameter reflects the different investment of photosynthates between the shoot and root compartment of the plant and can be affected by different biological and non-biological factors.

AM symbiosis generally lowers the root-to-shoot ratio. The fungus improves plant P uptake; and, in turn, it absorbs a significant proportion of organic carbon from the host. As a result, AM symbiosis forces plants to develop more the photosynthetic organs than the roots (Feddermann et al., 2010). The opposite response obtained with PGP-HSF inoculation, indicates the lack of nutrient transfer from the fungus to the plant. Accordingly, previous works on the effects of PGP-HSF on *M. piperita* led us to exclude the involvement of the fungus on plant mineral nutrition and to hypothesize a saprophytic behaviour of PGP-HSF with a one-way transfer of sugars from the plant (Fusconi et al., 2010). Alternatively, the interaction with the fungus may affect the allocation of assimilates and favour the production of hormones or other chemical compounds which could alter plant physiology and be responsible for the observed plant growth promotion.

In vitro roots normally improve the water status of micropropagated plants by ameliorating the water uptake capacity of the plant. Increases in water status derive not only from the presence of a well-developed root system but also from the amount of roots relative to shoot biomass. Larger root-to-shoot ratios in tissue cultures, for example, are positively correlated with transpiration rate and shoot water relative content (Díaz-Pérez et al., 1995).

For in vitro initiation of roots, sweet basil shoots are usually cultured on MS media containing different concentrations of auxins (Siddique and Anis, 2007; Saha et al., 2010) which are known as key regulators of root growth and lateral root formation (Fusconi, 2014). A well-developed root apparatus relative to the shoot size as that obtained with PGP-HSF without the use of auxins, can improve the propagation of this species ameliorating its water status and plant survival during acclimatization and ex vitro transplantation.

Beneficial microbial inoculants can be employed also to stimulate the production of useful active plant metabolites. Tada et al. (1996) reported greater production of rosmarinic acid in hairy root cultures than in intact plants of *O. basilicum*. Rosmarinic acid is an important and commercially valuable active compound possessing important anti-inflammatory and antioxidant properties (Kintzios et al., 2003). Moreover significant improvement of the growth and essential oil yield has been reported after inoculation of *Ocimum* spp. with endophytic bacteria (Tiwari et al., 2010; Singha et al., 2013) and AM fungi (Copetta et al 2006). Therefore, after PGP-HSP inoculation, a larger production of essential oils in vitro is likely. The observed increase of root tips and root weight in the presence of the fungal endophyte PGP-HSF, could thus represent an interesting alternative to transformed hairy root cultures for improving the in vitro production of rosmarinic acid in sweet basil.

In the phylogenetic study of SSU rDNA regions both Bayesian and Maximum likelihood analyses produced the same topology; therefore only the Bayesian tree with both BPP and MLB values is shown (Fig. 3).

The new phylogenetic analysis of the SSU rRNA sequences confirmed the previously recognized collocation of PGP-HSF in the Sordariomycetes (Mucciarelli et al., 2002); in both MP and Bayesian trees, PGP-HSF was related to the Magnaporthaceae family with weak support (MLB = 55; BPP = 0.90). The Magnaporthaceae family together with PGP-HSF constitute a well-supported sister clade of Ophiostomatales (MLB = 82; BPP = 1.00). Further studies including other genomic regions will be needed to refine the phylogeny of this fungus and to achieve a better understanding of its ecological role in nature.

In conclusion, the use of PGP-HSF for in vitro propagation of sweet basil has shown to be a valid tool for a rapid clonal multiplication of this species when high genetic uniformity is required.

Moreover, the features of the plants co-cultured with PGP-HSF have shown to be compatible with a good adaptability of sweet basil after transplantation and prompted us to study the influence of the fungus on the production of essential oils.

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Literature Cited

Ahuja, A., Verma, M., and Grwal, S. (1982). Clonal propagation of *Ocimum* species by tissue culture. Indian J. Exp. Biol. *20*, 455-458

Begum, F., Amin, M.N., and Azad, M.A.K. (2000). In vitro clonal propagation of Holy Basil – *Ocimum sanctum* L. Plant Tissue Cult. Biotechnol. *10*, 31–37.

Begum, F., Amin, M.N., and Azad, M.A.K. (2002). In vitro rapid clonal propagation of *Ocimum basilicum* L. Plant Tissue Culture Biotechnol. *12*, 27-35.

Copetta, A., Lingua, G., and Berta, G. (2006). Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum* L. var. Genovese. Mycorrhiza *16*, 485–94 http://dx.doi.org/ /10.1007/s00572-006-0065-6.

Díaz-Pérez, C., Shackel, K.A., and Sutte, E.G. (1995). Effects of *in vitro*-formed roots and acclimatization on water status and gas exchange of tissue-cultured apple shoots. Amer. Soc. Hort. Sci. *120*, 435-440.

Feddermann, N., Finlay, R., Boller, T., and Elfstrand, M. (2010). Functional diversity in arbuscular mycorrhiza - the role of gene expression, phosphorous nutrition and symbiotic efficiency. Fungal Ecology *3*, 1–8 http://dx.doi.org/10.1016/j.funeco.2009.07.003.

Fusconi, A. (2014). Regulation of root morphogenesis in arbuscular mycorrhizae: what role do fungal exudates, phosphate, sugars and hormones play in lateral root formation Ann. Bot. *113*, 19-33 http://dx.doi.org/10.1093/aob/mct258.

Fusconi, A., Trotta, A., Dho, S., Camusso, W., and Mucciarelli M. (2010). In vitro interactions between *Mentha piperita* L. and a non-mycorrhizal endophyte: root morphogenesis, fungus development and nutritional relationships. J. Plant Interact. *5*, 179- 188 http://dx.doi.org/10.1080/17429140903487560.

Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics *17*, 754-755 http://dx.doi.org/10.1093/bioinformatics/17.8.754.

Katoh, K., Misawa, K., Kuma, K., and Miyata T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucl. Acids Res. *30*, 3059–3066 http://dx.doi.org/10.1093/nar/gkf436.

Kintzios, S., Makri, O., Panagiotopoulos, E., and Scapeti M. (2003). In vitro rosmarinic acid accumulation in sweet basil (*Ocimum basilicum* L.). Biotechnol. Lett. *25*, 405–408 http://dx.doi.org/10.1023/A:1022402515263.

Mucciarelli, M., Scannerini, S., Bertea, C., and Maffei, M. (2003). In vitro and in vivo peppermint (*Mentha piperita*) growth promotion by nonmycorrhizal fungal colonization. New Phytol. *158*, 579–591 http://dx.doi.org/10.1046/j.1469-8137.2003.00762.x.

Mucciarelli, M., Scannerini, S., Bertea, C., and Maffei, M. (2002). An ascomycetous endophyte isolated from *Mentha piperita* L.: Biological features and molecular studies. Mycologia *94*, 28–39. http://dx.doi.org/10.2307/3761843

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. *15*, 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Phippen, W.B., and Simon, J.E. (2000). Shoot regeneration of young leaf explants from basil (*Ocimum basilicum* L.), In Vitro Cell. Dev. Biol. Plant. *36*, 250-254. http://dx.doi.org/10.1007/s11627-000-0046-y

Rodriguez, R.J., White, J.F. Jr., Arnold, A.E., and Redman, R.S. (2009). Fungal endophytes: diversity and functional roles. New Phytol. *182*, 314–330 http://dx.doi.org/10.1111/j.1469-8137.2009.02773.x.

Saha, S., Ghosh, P.D., and Sengupta, C. (2010). An efficient method for micropropagation of *Ocimum basilicum* L. Indian J. Plant Physiol. *15*, 168-172.

Shahzad A., and Siddiqui, S.A. (2000). In vitro organogenesis in *Ocimum sanctum* L. – A multipurpose herb. Phytomorphology *50*, 27-35.

Shetty, K. (1997). Biotechnology to harness the benefits of dietary phenols: focus on Lamiaceae. Asia Pacific J. Clin. Nutr. *6*, 162–171.

Siddique, I., and Anis, M. (2007). Rapid micropropagation of *Ocimum basilicum* using shoot tip explants pre-cultured in thidiazuron supplemented liquid medium. Biol. Plant. *51*, 787-790 http://dx.doi.org/10.1007/s10535-007-0161-2.

Singha, R., Sonib, S.K., Patela, R.P., and Kalrab, A. (2013). Technology for improving essential oil yield of *Ocimum basilicum* L. (sweet basil) by application of bioinoculant colonized seeds under organic field conditions. Ind. Crops Prod. 45, 335– 342 http://dx.doi.org/10.1016/j.indcrop.2013.01.003.

Smith, S.E., and Read, D.J. (2008). Mycorrhizal symbiosis, 3rd edn. (London, UK: Academic Press). pp.139.

Stamatakis, A. (2006). RAxML-VI-HPC: Maximum Likelihood-based Phylogenetic Analyses with Thousands of Taxa and Mixed Models, Bioinformatics *22*, 2688–2690 http://dx.doi.org/10.1093/bioinformatics/btl446.

Tada, H., Murakami, Y., Omoto, T., Shimomura, K., and Ishimaru, K. (1996). Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum*. Phytochemistry *42*, 431–434 http://dx.doi.org/10.1016/0031-9422(96)00005-2.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. *30*, 2725-2729 http://dx.doi.org/10.1093/molbev/mst197.

Tiwari, R., Kalra, A., Darokar, M.P., Chandra, M., Aggarwal, N., Singh, A.K., and Khanuja, S.P.S. (2010). Endophytic bacteria from *Ocimum sanctum* and their yield enhancing capabilities. Curr. Microbiol. *60*, 167-171 http://dx.doi.org/10.1007/s00284-009-9520-x.

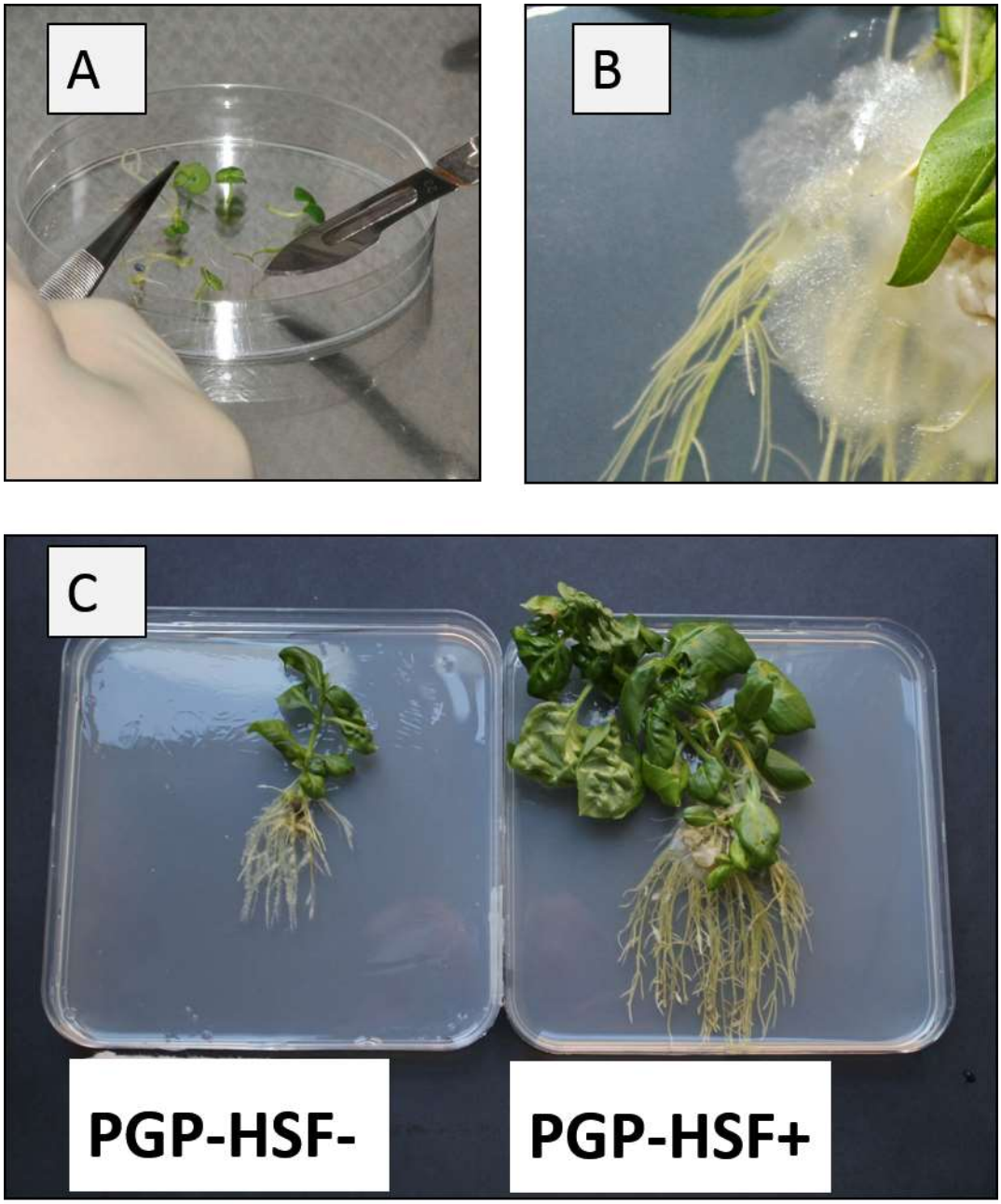

Figure 1: A: shoot cuttings of *Ocimum basilicum* L. B: mycelium of the fungal endophyte PGP-HSF in contact with sweet basil roots into the MS medium. C: two 35-days old plants of sweet basil, grown with PGP-HSF (on the right) and without (on the left).

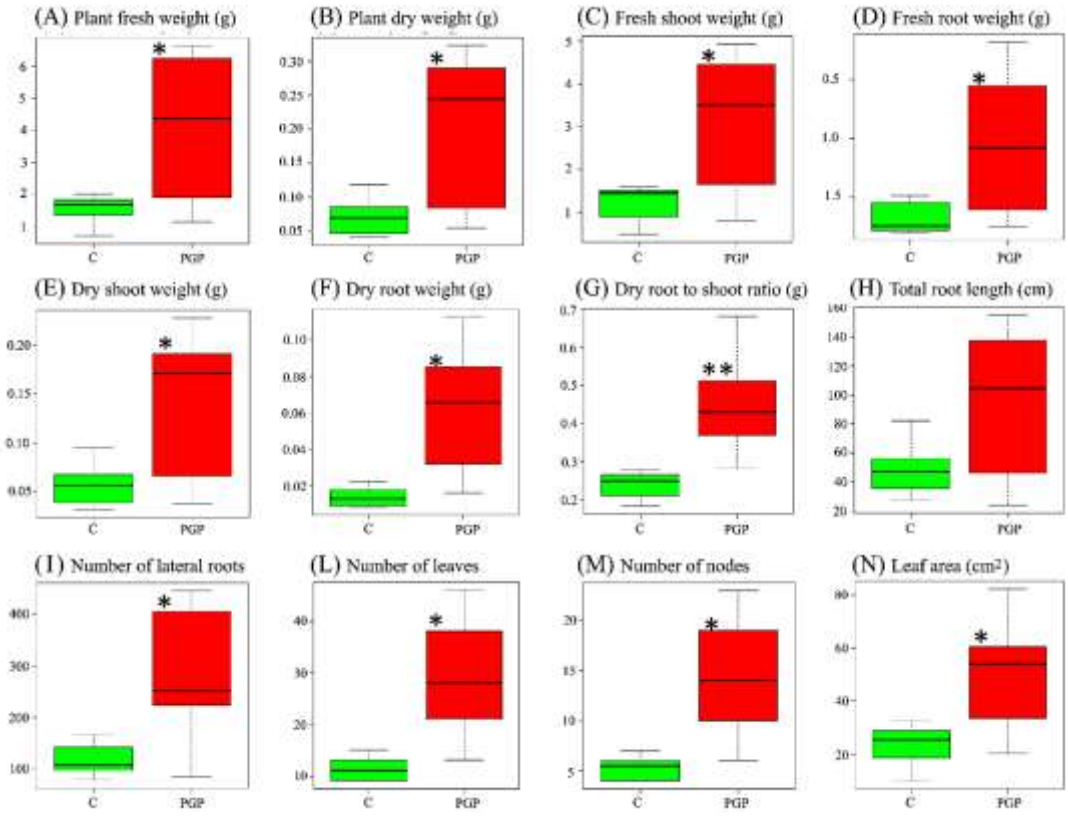

Figure 2. Sweet basil response to PGP-HSF after 35-d in vitro inoculation. Box plots show significant differences between plant growth parameters in controls (uninoculated: C) and with PGP-HSF (inoculated: PGP). Asterisks on the top of the box indicate treatment difference from the control based on t-test at $p<0.05$ * and $p<0.01$ **.

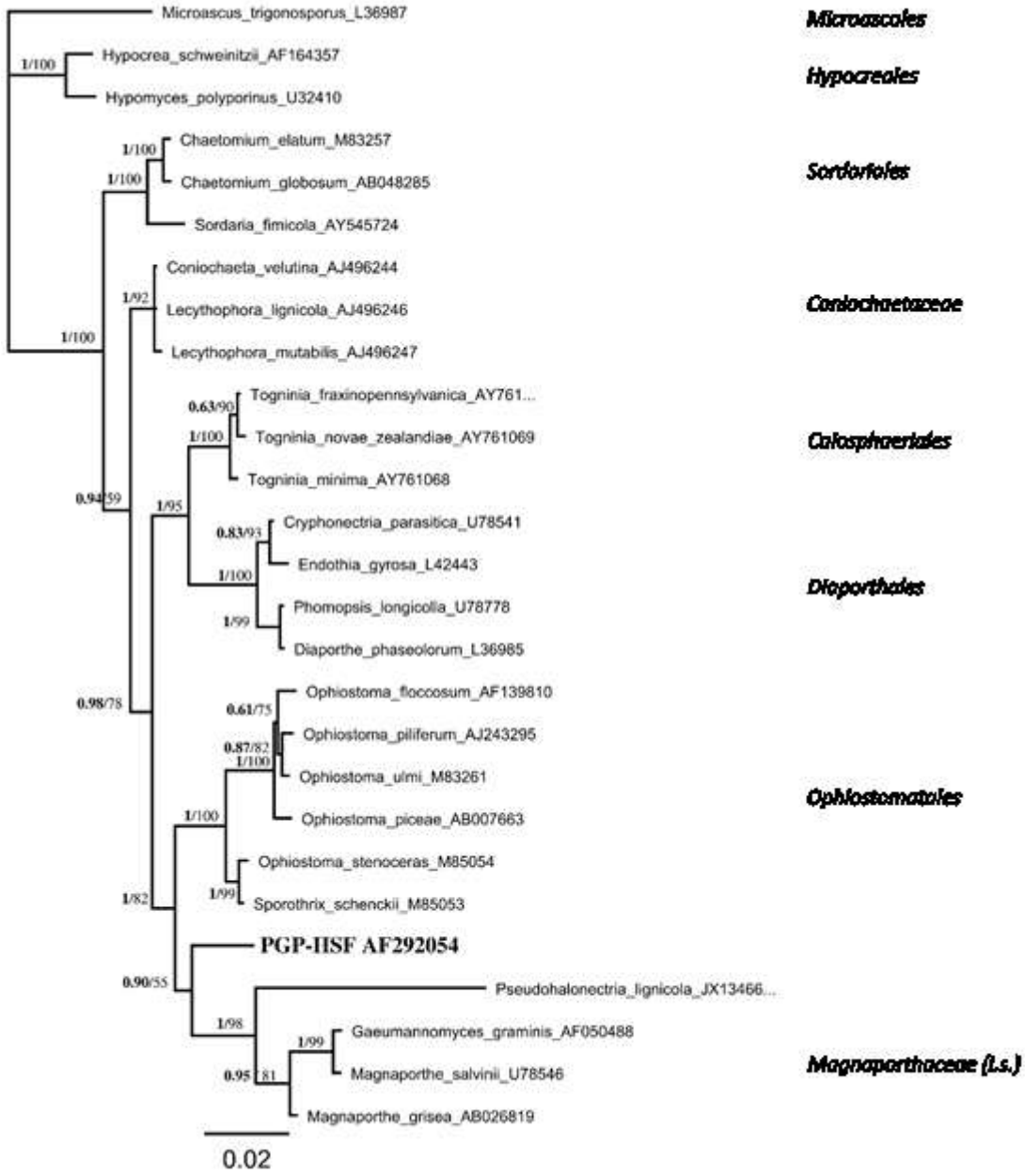

Figure 3. Bayesian phylogram obtained from 18s partial sequences. *Microascus trigonosporus* was used as outgroup taxon. Only BPP values over 0.50 (in bold) and MLB values over 50% are given above clade branches. PGP-HSF is in bold.