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ORIGINAL RESEARCH PAPER

Arbuscular mycorrhizae formed by *Penicillium pinophilum* improve the growth, nutrient uptake and photosynthesis of strawberry with two inoculum-types

Yongqiang Fan · Yushi Luan · Lijia An · Kun Yu

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Abstract *Penicillium pinophilum* was isolated from the soil in a commercial strawberry field. The strain readily formed arbuscular mycorrhizae (AM) with the roots of strawberry 'Zoji' (Fragaria × ananassa Duch. CV.) when plants were inoculated with either fresh cultured hyphae or root/soil mixtures. Fresh hyphae, however, resulted in higher amounts of colonization than root/soil inoculum. Compared with uninoculated strawberries, inoculation increased plant dry weight by 31%, as well as nitrogen content (47%), phosphorus content (57%), and photosynthetic rate (71%). AM inoculation also shortened the blossom and ripening date by 3 and 4 days, respectively. This is the first report of a *P. pinophilum* strain resulting in mycorrhiza with strawberry roots. The significant advantages of this strain are that it is easy to culture and inoculation of plants results in significant growth benefits that may be useful in strawberry production.

Keywords Arbuscular mycorrhizae · *Penicillium pinophilum* · Photosynthesis · Physiology improvement · Strain isolation · Strawberry

Y. Fan · Y. Luan (✉) · L. An · K. Yu
School of Environmental and Biological Science and Technology, Dalian University of Technology, Dalian, P.R. China
e-mail: luanyushi@yahoo.com.cn

Introduction

Mycorrhizae are symbiotic associations between plant roots and fungi that occur widely natural communities (Read 2000). The most common type is formed by the arbuscular mycorrhizal fungi (AMF), which colonize the roots of over 90% of plant species (Gadkar et al. 2001). AMF can influence plant community composition by differentially affecting the growth of plant species. Arbuscular mycorrhizal fungal symbiosis is a highly dynamic interaction affecting many aspects of the host plant physiology, including an enhanced uptake of phosphorus (P) and nitrogen (N) (Nowak 2004), and increased photosynthetic capacity (Borkowska 2002).

Strawberries are an important crop in northern areas of China (Gao 2000). Although commercially grown strawberries might benefit from mycorrhizae, producing large amounts of fungal inocula is difficult and time consuming. Recently, methods of inducing mycorrhizae by inoculating plants with AMF have been investigated. The two key requirements for mycorrhizae development are superior AMF strains and appropriate inocula. Since most AMF are obligate biotrophes in nature, they are difficult to culture in vitro, and this is a major obstacle to their practical use (Srivastava et al. 1996). A traditional method of producing large amounts of inoculum is to culture the fungi on roots in soil (Mosse and Hepper 1975), but this method is very tedious and time-consuming. An alternative approach is to screen AMF to identify

types that can be easily cultured under axenic conditions, so that fresh-hyphae can be grown without plants and directly utilized as inoculum.

The objective of this work was to identify easily cultured AMF species, and compare the efficacy of soil-root and fresh-hyphae inoculum on the growth, nutrient uptake, and photosynthesis of strawberry plants.

Materials and methods

Arbuscular mycorrhizal fungi (AMF) isolation and species identification

Soil was collected from a commercial strawberry field and AMF were isolated using a sucrose centrifugation technique (Rajni and Mukerji 2002). The sieved spores were cultured and germinated in distilled water, and then transferred onto modified Melin-Norkans (MMN) media for the hyphal growth. Fungal species were identified from morphological characteristics. Molecular identification of the isolates was performed using the sequence data of the Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA, with the primers pair ITS1 and ITS4 (White et al. 1990) in the PCR condition as previously described (Jakucs et al. 2005). The sequencing of the ITS region was performed by TaKaRa Biotechnology (Dalian) Co., Ltd.. The sequence alignment was conducted by the Nucleotide Blast Programme of NCBI to search for the resembling AMF.

Strawberry plant material and inoculation

Plants of 'Zoji' strawberry (*Fragaria* × *ananassa* Duch. CV.) were propagated by tissue culture, then grown in sand for 1 month. Plants were then transferred to plastic pots containing 3 kg of autoclaved original field soil (0.11 MP, 121°C, 1.5 h). Two types of mycorrhizal fungus inoculum were compared. The first was a root-soil mix produced by Mosse's method (1975) which consisted of spores, soil, hyphae, and infected clover roots. The second inoculum was fresh-hyphae cultured on liquid MMN media. Each pot received either 7 g soil-root inoculum containing approx 500 spores, 1 g fresh-hyphae inoculum, or the same weight of autoclaved growth media as the soil root inoculum (uninoculated, CK). Each treatment was replicated 15 times. The pots were placed in a greenhouse under natural light, where no temperature controlling equipment was available. The average day/night temperature was 25/16°C.

Plant growth and mycorrhizal colonization measurement

After 60, 80, and 100 days in the greenhouse, four plants were harvested and the leaves, stems, and roots were separated. The fresh weights (FW) of each part was recorded. Tissues were then dried at 75°C for 48 h and their dry weights (DW) were recorded. A portion of fresh roots were carefully washed and stained (Phillips and Hayman 1970). Mycorrhizal colonization percentage was determined using a grind-line intersect method. Three plants of each treatment were reserved to determine bloom and fruit ripening data, and fruit yields.

Physiological measurements

Dried plant tissues, 6 mg, were wet-combusted (Sune and Åsa 1990) and total tissue N concentrations were measured by the Kjedahl method, and phosphorus (P) concentration was determined at 880 nm using molybdenum/antimony colorimetry (Wang et al. 2004).

Stomatal conductance (Gs), transpiration rates (E), and photosynthetic rates (Pn) were measured by a CIRAS-2 Photosynthesis System from 10:00 am to 11:30 am on September 28, 2007. Fifteen leaves of each treatment were selected for measuring. Reference CO_2 concentration was 375 μmol mol^{-1} and cuvette air temperature ranged from 28 to 30°C.

Results

One strain of fungus *Penicillium pinophilum* was identified by morphological characteristics after being isolated from soil. The ITS sequence was obtained with a length of 544 bp, which had been deposited at Genbank (accession no. EU277738). The alignment result showed it had very high homology with reported *P. pinophilum* (EF211128, 539/543, 99%).

The roots of plants inoculated with *P. pinophilum* were infected 60 days post-inoculation (Table 1). The fresh-hyphae resulted in 97% higher colonization rates than the soil-root inoculum. Mycorrhizae was absent from uninoculated control plants.

The DW of the roots, stems, leaves, and whole plants were greater for AMF inoculated plants than CK plants (Fig. 1a), and plants inoculated with fresh-hyphae had higher dry weights than those with soil-root inoculum. Differences in DW between the three treatments were significant after 80 and 100 days. Effects on plant fresh weights were similar to DW effects (data not shown).

Compared to the CK plants, the blossom date was shortened by 3 days and the fruit ripening date was shortened by 4 days with the inoculated plants. However, there were no significant differences for fruit yields in comparison with CK strawberries, and mean yield across all treatments was 126.5 g/plant.

The concentrations of N and P in the roots, leaves, stems, and whole plants were increased by the AMF inoculation compared to the controls (Fig. 1b, c). The fresh-hyphae inoculum usually resulted in higher concentrations of N and P than the soil-root inoculum. The inoculation treatments tended to have a greater effect on the concentrations of P than N; differences in *P* levels were significant 80 days after inoculation.

AM inoculation also affected photosynthetic parameters (Table 1). Compared to CK plants, plants receiving the fresh-hyphae and root-soil inocula had higher Pn, Gs, and E levels. However, there were no significant differences between the two inoculum types.

Discussion

The strain of AMF isolated from a strawberry field soil was identified as *P. pinophilum* through morphological and molecular methods, which was a new strain of *P. pinophilum*. The results of the synthesis experiment with the strawberry plants showed the strain could form typical structures of mycorrhizae in the roots. It was reported that *P. pinophilum* had the ability to mineralize phosphate and provide biocontrol of plant diseases (Alagesaboopathi and Subramanian 2007; Mehana and Abdul 2002). However, there were no reports *P. pinophilum* strains could form mycorrhiza with strawberry.

The isolated strain of *P. pinophilum* could be easily cultured in the laboratory. This method of producing fresh-hyphae for use as inoculum was more convenient and less time-consuming than producing soil-root inoculum. Although no spores were observed in the fresh hyphae, this inoculum source colonized the roots of the host-plant more rapidly than soil-root inoculum. The fresh hyphae also had greater effects on plant physiology and growth than the soil-root inoculum.

Our results confirm the fact that AM inoculation can enhance plant growth and nutrient uptake (Koide and Mosse 2004; Borkowska 2002; Henrike et al. 2007; Kaya et al. 2003). AM inoculation had modest effects on N and P accumulation between 60 and 80 days after inoculation, but more dramatic effects between days 80 and 100 (Fig. 1b and c). The strawberry plants entered a more active growth stage towards the end of the experiment and have a greater demand for nutrients at this time. These observations confirm that AMF enhanced the nutrition of strawberry plants, and that an effective time for inoculation with *P. pinophilum* is about months before the vegetable growth stage of plants.

AMF inoculation did not significantly affect fruit yields compared with CK plants, the main reasons for which may be the plants in this experiment were relatively young and their flower and fruit production was somewhat unstable. Meanwhile, we observed more desirable flavour in fruit from inoculated plants but scientific evaluations were not conducted. A longer

Table 1 Effect of inoculation treatment on root AM colonization, and leaf photosynthetic rate (Pn), stomatal conductance (Gs) and transpirations rate (E) of 'Zoji' strawberry plants 60 days after inoculation

Inoculation treatment	AM colonization (%)	Pn (μmol/m^2 s)	Gs (mmol/m^2 s)	E (mmol/m^2 s)
Control	0^{c}	8 ± 0.1^{b}	119 ± 5^{b}	2.8 ± 0.4^{b}
Soil-root	36 ± 8^{b}	11 ± 0.2^{a}	149 ± 26^{ab}	3.2 ± 0.1^{ab}
Fresh-hyphae	71 ± 5^{a}	13 ± 2.4^{a}	156 ± 7^{a}	3.5 ± 0.4^{a}

Different letters denote significant differences between means within columns determined by the Duncan's test ($P < 0.05$). Values for AM colonization are means of 30 observations ± SD, others are means of four observations ± SD

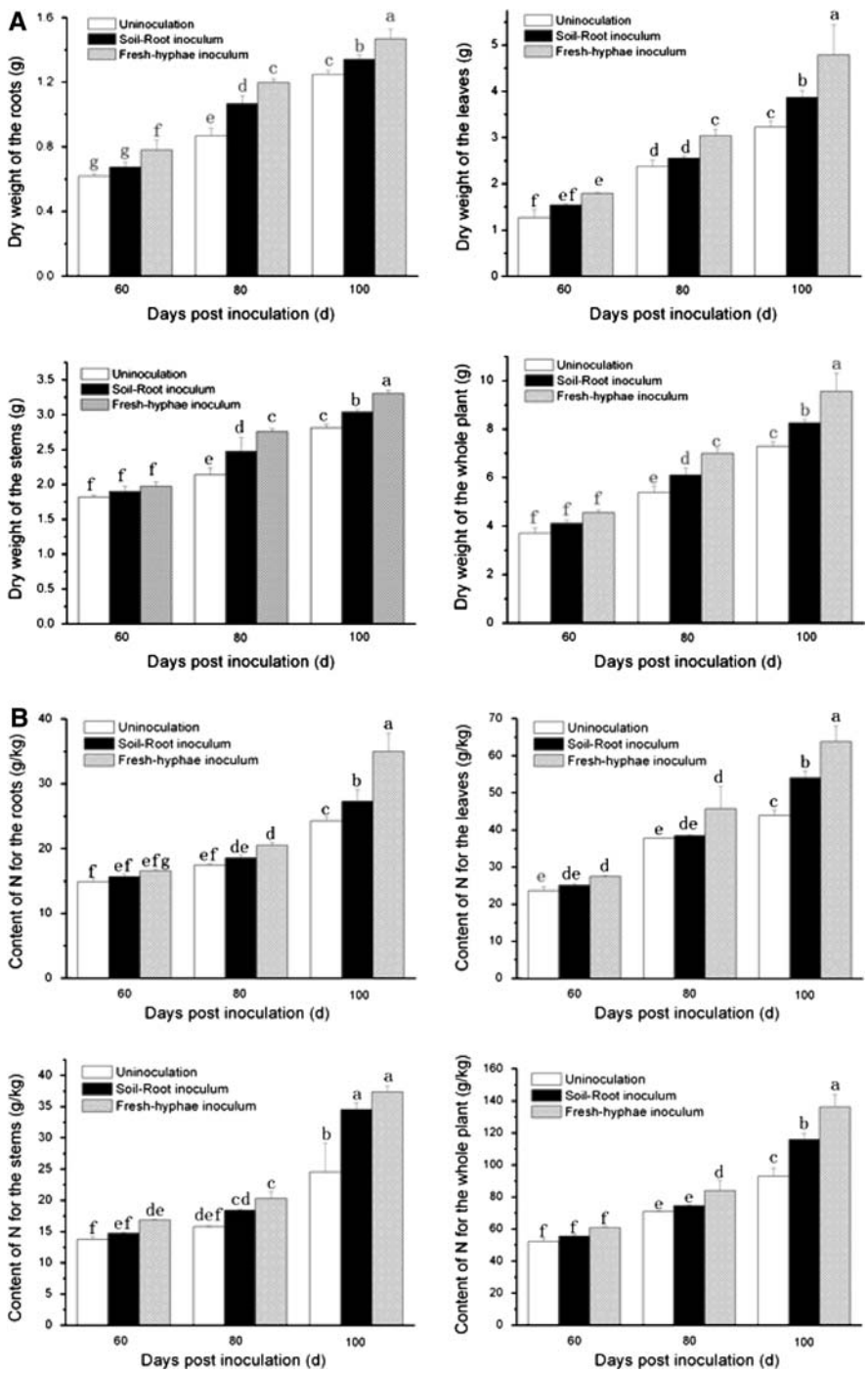
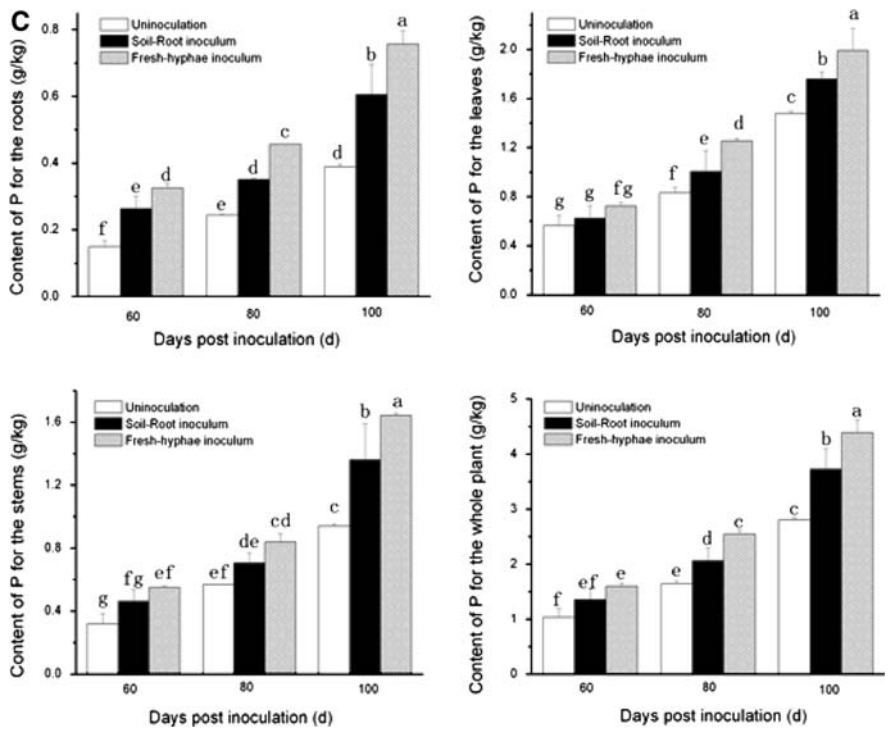


Fig. 1 Effect of *P. pinophilum* on the dry weights (**a**), nitrogen (**b**) and phosphorus (**c**) contents of 'Zoji' strawberry plants and plant parts with three treatment: uninoculate □; soil-root inoculation ■; fresh hyphae inoculation ■. Different letters denote significant differences between means as determined by the Duncan's test ($P < 0.05$). Bars represent SD (n = 4)

study would be needed to determine how AMF inoculation affects fruit production and quality. A longer duration study would also help to confirm our initial observations that inoculation shortens the fruit ripening time and improves fruit flavour.

The results in this study indicate AM strawberry plants had higher Pn, E, and Gs than non-AM plants. This is consistent with some previous reports (Borkowska 2002). We suspect that the primary benefit from AM colonization is an improvement in host

Fig. 1 continued



plant mineral nutrition, and that altered photosynthetic characteristics may be secondary effects of improved nutrition.

In summary, this work showed that a strain of *P. pinophilum* isolated from the soil forms typical AM structures within the roots of strawberries. Fresh hyphae appear to be a more effective inoculum than root-soil material, although both inocula result in colonization. Root colonization resulted in enhanced plant growth, nutrient uptake, and photosynthesis characteristics of 'Zoji' strawberry. More work is needed to determine how AMF inoculation affects fruit production and flavor.

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