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Effect of vesicular arbuscular mycorrhizae on growth and saponin accumulation in *Chlorophytum borivilianum*

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ABSTRACT: Safed musli (*Chlorophytum borivilianum*) contains pharmacologically important steroidal saponins that have attracted pharmacological societies and researchers worldwide. To estimate the beneficial effects of different vesicular arbuscular mycorrhiza (VAM) and *C. borivilianum* symbiotic association we studied phosphorus uptake, root carbon, spore build up, root dry mass, and saponin accumulation in different harvesting periods. In the VAM colonized roots studied, a significantly higher spore build-up was found in *Glomus intraradices* and *Glomus mosseae* than in the *Glomus fasciculatum* or non-colonized treatment at the critical growth stage of the species (90 days). However, all the mycorrhizal treatments showed significant increase in phosphorus uptake and root carbon percentage. The results reveal that mycorrhizal fungi substantially enhance the saponin content of *C. borivilianum*, depending upon the type of VAM fungi supplied. *G. mosseae* contributed 5 fold (0.56 g to 2.8 g/plant) enhancements in saponin accumulation followed by *G. intraradices* (0.56 g to 2.7 g/plant) in comparison to non-mycorrhizal plants. After 270 days, saponin content and root growth in all the mycorrhizal inoculated plants was found to be greater than in non-mycorrhizal material. The present study is a first report of an increase in secondary metabolite accumulation and root growth enhancement in *C. borivilianum* as an effect of mycorrhizal inoculation.

KEYWORDS: safed musli, *Glomus mosseae*, *Glomus intraradices*

INTRODUCTION

Chlorophytum borivilianum Santapau & R.R. Fern., commonly known as safed musli, is an endangered traditional medicinal plant which belongs to the family Liliaceae. In India, *C. borivilianum* is mainly distributed in southern Rajasthan, north Gujarat, and western Madhya Pradesh[1]. Among all the species of *Chlorophytum* present in India, *C. borivilianum* produces the highest yield of roots and has the highest saponin content[2]. The root has spermatogenic properties and is found useful in curing impotency[3]. It acts as an immunomodulator, aphrodisiac, antidiabetic[4], anticancer[5,6], anti-fungal, hepatoprotective, antimalarial, and rejuvenator sedative drug[2,7]. In a search for bioactive steroidal saponins, a phytochemical investigation on *C. borivilianum* roots led to the isolation and structure elucidation of four new furostane-type steroidal saponins called borivilianosides A-D[8,9]. In recent years, saponins are gaining popularity as substitutes for Viagra[10]. With the growing importance of this natural drug, overexploitation of *C. borivilianum* in its natural habitat makes the species threatened and hence its cultivation is being increasingly practised.

Vesicular arbuscular mycorrhiza (VAM) fungi are the most ancient and widespread obligate symbionts[11]. Mycorrhizal fungi are well adapted for nutrient acquisition. Their small size allows them to act as microscopic pipelines that can transport carbon and minerals to and away from the plant[12]. They improve the growth and biomass of a wide host range and are efficient phosphate solubilizers and transporters. They also play a significant role in reestablishing and conserving the endangered plant under a fragile environment. Indigenous medicinal plants show varied mycorrhizal colonization. However, the use of pesticides and fertilizers for enhancing growth affects the quality of the medicine and this can be mitigated by inducing mycorrhizal associations[13]. The present study has been made to increase the saponin concentration, which is the major bioactive compound in the tubers of safed musli through mycorrhizal fungi. Infection of mycorrhizae, phosphorus uptake, and enhancement in the root biomass were studied at different stages of harvesting.

MATERIALS AND METHODS

The experiment involved a pot trial. The objective was to enhance the VAM endophyte population in different harvesting periods using loamy sand soil collected from barren lands of Jodhpur under arid environment. The preliminary analysis of soil indicated pH 7.9, EC 0.13 dS/m, organic C content 0.03%, Olsen's P 3.5 mg/kg, organic P 60.2 mg/kg, and total P 134 mg/kg. Earthen pots (50 cm $\times$ 30 cm) of 25 kg capacity were filled with 20 kg sieved (2 mm) soil. The experiment consisted of a randomized complete block design with 30 replicates. Pure cultures of mycorrhizal inoculum were purchased from COA, Bangalore. Four treatments were carried out viz. inoculated with *Glomus fasciculatum* (Thaxter) Gerd. & Trappe (CG 132), *Glomus intraradices* N.C. Schenck & G.S. Sm. (CG 133), *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (CG 134), and control (without AM inoculum). Inoculum containing 1500 spores (75 spores/kg soil) was placed in each VAM treatment. Healthy tubers of *Chlorophytum borivilianum* were purchased from the Rajasthan Seed Corporation, Indore, India and tubers weighing 8–10 g were sown in each pot. Plants were grown under controlled conditions with 80% RH, day/night temperature of 25/15 °C, and a photoperiod of 16 h at a photosynthetic photon flux density of 460–500 µmol $m^{-2}s^{-1}$. No manure or fertilizers were applied at any stage of plant growth. The plant materials were harvested after 45, 90, 180, and 270 days. Fresh and dry mass of roots were measured.

To assess the root colonization by VAM fungi, the standard staining technique of Phillips and Hayman[14] was followed. The roots of plants were cleared with 10% (w/v) KOH at 90 °C for 12–15 min in a water bath, rinsed three times, stained in 0.1% trypan blue (made in lacto phenol) at 90 °C for 3–5 min, and mounted in lactic acid:glycerol (1:1). The recovery and quantification of VAM fungal propagules for spore build-up studies were done in different harvesting periods. Spores of different species were extracted by the wet sieving and decanting technique of Gerdemann and Nicholson[15]. Total spore numbers of mycorrhizal fungi in the soil samples at the different harvesting intervals were estimated by the method of Gaur and Adholeya[16]. All the spores were examined using a Medilus-20 TR compound microscope. The endophyte dependency (ED), indicating how much a plant species depends on AMF for its growth, was determined according to Ref. 17. An endophyte dependency > 0 means that the plants benefited from VAM. Organic carbon was estimated by the method of Walkley and Black[18] using 1 N potassium dichromate and back titrated with 0.5 N ferrous ammonium sulphate solution. Available phosphorus in the soil was determined by extraction with 0.5 M sodium bicarbonate for 30 min[19].

Saponin content in the roots was estimated at different harvesting periods[20] by using microwave-assisted extraction instead of refluxed extraction. The root powdered material was defatted using petroleum ether and extracted with ethanol, chloroform, and butanol in 20-ml closed vials, which were placed in a mechanically modified microwave oven (ETHOS 1600, Milestone, Sorisole, Italy) and irradiated at 2450 MHz for 10–20 min. The solvent temperature was kept constant at 60 °C using an automatic temperature control device (ATC-FO, Milestone) submerged in a solvent containing vessel. Twelve sample TFM (a thermally resistant form of Teflon) vessels were used at a time, with pressure and temperature monitoring capabilities, in a MPR-600/12S rotor (Milestone). The microwave power was limited to 300 W. After cooling to room temperature, the extract was collected and kept at -20 °C until analysis. Finally, the saponin was quantified using a colorimetric reaction mixture: dry powder (0.2:1 mg) was dissolved in acetic acid (1.5 ml) to which sulphuric acid (1 ml) was added. The absorbance at 530 nm of the reaction product was determined after 15 min incubation at room temperature[20].

All the parameters were analysed by one way ANOVA. Mean comparisons were determined by Waller-Duncan at $p \leqslant 0.05$. Statistical tests were performed with SPSS version 10.

RESULTS AND DISCUSSION

Arbuscular mycorrhizae with arbuscules, which are the structural and functional criterion of the symbiosis, were observed in the case of all inoculated plants. No other root endophytes were detected in the investigated material. The root yield (dry wt.) did not differ significantly within the treatments of *G. intraradices* and *G. mosseae* but was found very significant in comparison to *G. fasciculatum* as well as non-inoculated plant material (Fig. 1a). Among all the VAM inocula a higher spore build up was found in the case of *G. intraradices* and *G. mosseae* than with the *G. fasciculatum* and non-inoculated treatments in the critical growth stage of the species (90 days). Plant materials inoculated with *G. mosseae* and *G. intraradices* showed 48% and 45% root C, respectively. All the mycorrhizal treatments showed a significant increase in P uptake and in percentage of root C than the control. However, plants colonized

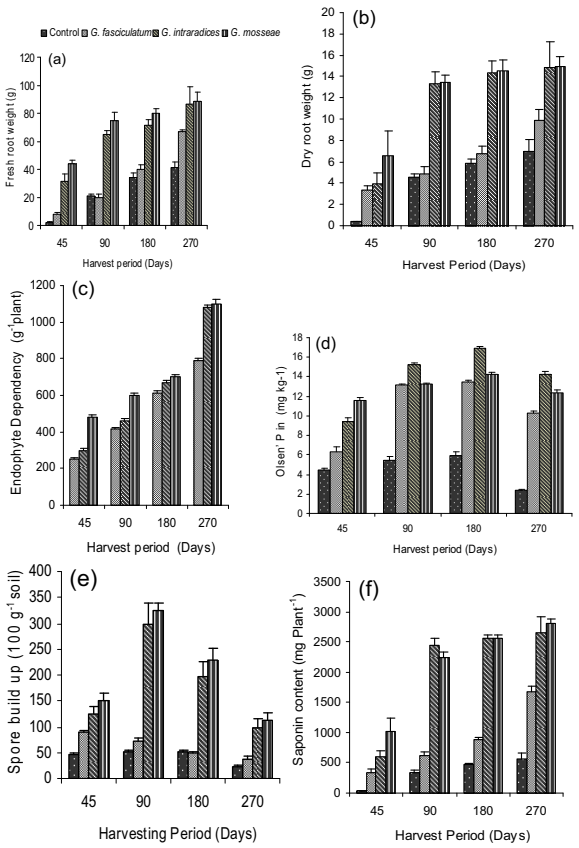

Fig. 1 Effect of VAM on (a) fresh root weight (b) dry root weight (c) endophyte dependency (d) P uptake (e) spore build-up (f) saponin accumulation at different harvesting periods. Bar indicates mean ± SE of 30 replicates.

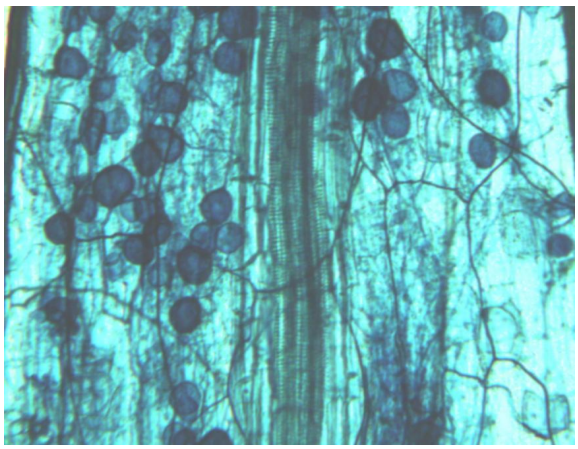

Fig. 2 *G. mosseae* spore infection in root of *C. borivilianum*.

Fig. 3 Root yield after final harvest with different inoculum. Bar indicates mean ± SE of 30 replicates ($p \leqslant 0.05$).

by *G. intraradices* were found to be responsible for the optimum P uptake after 180 days (Fig. 1d). Endophyte dependence revealed that all the investigated plant materials inoculated with mycorrhizal spores were strongly dependent on VAM for their growth (Fig. 2). However, the *C. borivilianum* roots infected by *G. intraradices* and *G. mosseae* did better than the *G. fasciculatum* colonized roots after 270 days. The increase in root dry mass and carbon percentage during the final harvesting period confirmed the increase in endophyte dependence in the inoculated plants (Fig. 3).

In recent years, the use of microwaves for the extraction of constituents from plant material has shown much potential. Conventional techniques for the extraction of active constituents are time- and solvent-consuming, thermally unsafe, and the analysis of numerous constituents in plant material is limited by the extraction step. In all the mycorrhizal treatments, the saponin concentration in roots was found to be significantly higher ($p \leqslant 0.05$) than the non-inoculated plant materials. The saponin accumulation also increased with the age of the plant. Roots harvested at maturity (270 days) showed the highest accumulation (Fig. 1f). The results show that mycorrhizal fungi substantially contribute to the saponin content in safed musli, depending upon the type of AM fungi supplied. *G. mosseae* caused a 5-fold (0.56–2.8 g/plant) increase in saponin accumulation followed by *G. intraradices* (0.56–2.7 g/plant). The harvested plant materials colonized by *G. fasciculatum* had a significantly poor saponin enhancement compared to the other two VAM species. In addition, the early harvesting of plant materials was proven to be detrimental to the production of saponin.

In our study, it was observed that the degree to which *C. borivilianum* roots were colonized by VAM

varied among microcosms inoculated with different mycorrhizal species. Arbuscular mycorrhizal fungi increase the host plant fitness by increasing the uptake of minerals such as P that are relatively immobile in soils[21]. In our studies, we also found that Olsen's P increased significantly in the colonized plant materials compared to the non-mycorrhizal plants. This may be because VAM fungi help to increase nutrient uptake by increasing the surface area of the plant absorptive system (roots) and exploring soil by extraradical hyphae beyond the root hair and P-depletion zone. The absorbed P is then converted to polyphosphate granules in external hyphae and passed to the arbuscule for transferring to the host plant[22]. Higher root biomass production in mycorrhizal plants compared to nonmycorrhizal plants has been frequently reported[23]. Similar results were seen in our study. Mycorrhizal spore build-up in the case of safed musli after 90 days was found to be significantly higher than after 180 or 270 days.

Our experiment is first of its kind to enhance the saponin accumulation in *C. borivilianum* by VAM species and found significant increase in all the mycorrhizal plant material. The results clearly demonstrated that safed musli should be harvested after 270 days as the saponin concentration is increased in the later stage of plant growth in the case of plants colonized by VAM. The exact mechanism by which the VAM symbiosis promotes such an effect remains to be elucidated. It could be thought that mycorrhizal plants were simply better hydrated than nonmycorrhizal ones due to direct fungal water uptake and its transport to the plant[24]. However, it is unlikely that this is the only mechanism involved. But VAM species as biofertilizers can be used extensively for the cultivation of medicinal plant species like *C. borivilianum* for the production of their active secondary metabolites on the large scale.

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