

Development of Arbuscular Mycorrhizal Spore Production in Hydroponic Culture on Leaf Lettuce (*Lactuca sativar var crispa* L.)

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ABSTRACT

*This study aimed to assess the suitability of a nutrient liquid solution for the production of *Glomus verruculosum* on leaf lettuce, a highly-colonized horticultural crop that is a host of arbuscular mycorrhizal (AM) fungi for inoculum production. A hydroponic culture was developed to allow nursery production of an AM horticultural crop. A suitable host of leaf lettuce cultivars was selected by an initial experiment. The red-leaf variety was found to be the best host because it exhibit maximum amount of root infection. In a second experiment, 2 nutrient media; modified Long Ashton (LANS) and modified Hoagland medium (HNS) were compared for culturing AM fungi on leaf lettuce. After a pre-culture period of 4 weeks with 50 spores in sand substrate, the plants initially infected with AM fungi and irrigated with both LANS and HNS were not significantly different. At 4 weeks after transplanting in the hydroponic culture, the total percentage of root colonization was found to be significantly greater in HNS medium than in LANS medium. In a third experiment, 4 nutrient media (solution-I, solution-II, solution-III and solution-IV) of HNS were used to compare root colonization, plant growth and spore production. The solution was changed every week by a deep water culture technique. At harvest time, plants in a solution-I with containing of NO_3^- as the N source and insoluble Ca_3PO_4 and $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ had a higher percentage of mycorrhizal colonization and spore population than those using other nutrient media.*

Keywords: Arbuscular mycorrhiza, Leaf lettuce, Hydroponic culture, Spore production

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are soil-born fungi that establish an obligate, mutually symbiotic relationship with approximately 90% of all vascular plants (Gerdemann, 1968). They provide certain benefits of plant mineral nutrition and general plant health (Gianinazzi et al., 2003) and have a high potential for improving agriculture and land reclamation (Menge, 1984; Sylvia, 1990). Due to an ability to increase the nutrient uptake and water transport of infected plants, AM inoculum is frequently used in sustainable agriculture (Sylvia, 1990). Traditionally, mass production of AM inoculum has been carried out in pot culture because of its availability, low cost and general suitability (Jarstfer and Sylvia, 1993). However, since it is time-consuming, bulky and frequently contaminated by pathogens, the production of a pure inoculum has recently been established by employing soil-free methods. Soilless cultures including static hydroponics (Hawkins and George, 1997), nutrient film technique (NFT) (Lee and George, 2005), aeroponics (Asif, 1997) and in vitro culture with transformed root (Pawlowska et al., 1999) are some of the well-known techniques. It is now widely accepted that soilless cultures can provide a high-quality inoculum with large-scale production (Millner and Kitt, 1992).

The AM inoculum is normally quantified by AM spore production which is affected by root colonization (Gaur et al., 1998) and the root colonization can be diminished by high levels of available phosphorus (P) and calcium (Ca) (Sieverding, 1991) in the plant grown media. There are reports that the hydroponics culture was performed in the modified nutrient media based on Long Ashton nutrient solution (LANS) that has been used previously as a nutrient solution for sand culture of mycorrhizal plants (Hawkins and George, 1997) and Hoagland nutrient solution (HNS) (Dugassa and Grunewaldt-Stocker, 1995) at half strength with a low or no P content (Gaur and Adholeya, 2000), with rock phosphate (Elems and Mosses, 1984), with solid CaSO_4 and rock phosphate (Warner et al., 1985; Sieverding, 1991) and a solution containing an MES buffer (Millner and Kitt, 1992).

The soil-based method is a commonly-used technique for commercial AM inoculum production except for hydroponic culture using corn as a host plant. Leaf lettuce, a highly-colonized horticultural crop, is a good potential host of AM fungi because of its short life cycle and abundant root system. Therefore, the objective of this study was to evaluate the effects of plant nutrient solutions on leaf lettuce growth, root colonization and spore production of AM fungi in hydroponic culture. At the conclusion of this study, further developments of soilless culture are proposed to be used for the mass production of lettuce-AM fungi.

MATERIALS AND METHODS

Preparation of AM fungal inocula

The spores of *Glomus verruculosum* were collected from lettuce rhizosphere soil and isolated, by using a sucrose gradient procedure (Danny and Brenda, 2006). The AM fungi species identity was verified by comparison with

reference cultures obtained from the International culture collection of arbuscular & vesicular-arbuscular mycorrhizal fungi (INVAM) (Gai et al., 2006). An indigenous *G. verruculosum* was propagated by pot culture for one year in the greenhouse (Simpson and Daft, 1990). At senescence, the above-ground plant material was removed and the substrate allowed to dry. The roots were finely chopped and the dried root/sand mixture was thoroughly mixed to obtain a homogenous inoculum (Gaur and Adholeya, 2002).

Seedling preparation

Seeds of leaf lettuce cultivars (green leaf, red leaf, granada lettuce and oak leaf) were surface-sterilized with 3% (v/v) H₂O₂ for 5 min. Seeds were then washed 5 times with sterile distilled water and incubated for germination on moist cotton layers in sterile Petri dishes (Gaur and Adholeya, 2000) at 25°C in a light room with 12 hr. photoperiods and a photosynthetic photon flux density of 800 μmol m⁻²s⁻¹ (Bio fluorescent lamp, Silverlight Plant Growth 6000K).

AM inoculation

AM spores were isolated by wet sieving and decanting (Brundrett et al., 1996). The collected spores were sterilized as described by Arnaud et al. (1996). One seedling was planted directly in a 30 mL pot filled with the sterilized moistened fine sand (Gaur and Adholeya, 2000) and 50 spores of *G. verruculosum* were inoculated (Ahmad and Raddad, 1995). The pots were placed in a light room under the same conditions as described above.

Nutrient media

The plants were supplied with various nutrient media. The Long Ashton nutrient solution (LANS) consisted of the following macronutrients (mM): Ca(NO₃)₂.4H₂O (2); NaH₂PO₄.2H₂O (0.0094); Na₂HPO₄.12H₂O (0.006); K₂SO₄ (1); MgSO₄.7H₂O (0.75); CaCl₂.2H₂O (2) and micronutrients (μM): H₃BO₃ (69); MnSO₄.4H₂O (10.4); ZnSO₄.7H₂O (1.2); CuSO₄.5H₂O (1.7); NaMoO₄.2H₂O (1.3) and FeEDTA (0.3) (Hawkins and George, 1997). The Hoagland nutrient solution (HNS) consisted of the following macronutrients (mM): Ca(NO₃)₂.4H₂O (5); KNO₃ (5); KH₂PO₄ (2); MgSO₄.7H₂O (2); and micronutrients (μM): MnCl₂.2H₂O (2); H₃BO₃ (10); ZnSO₄.7H₂O (1); CuSO₄.5H₂O (0.5); NiSO₄.6H₂O (0.2); CoCl₂.6H₂O (0.2); NaMoO₄.2H₂O (0.2) and NaFeEDTA (0.1) (Millner and Kitt, 1992). The pH of the media was adjusted to 6.8 using 1 N HCl or 1 N KOH. Additionally, four different plant nutrient solutions (Solution-I, II, III, and IV) modified from the Hoagland nutrient solution were applied. Solution-I was composed of Ca(NO₃)₂.2H₂O (40 mg/L) as N-source without P and plus insoluble Ca₃PO₄ (100 mg/L) and solid CaSO₄.2H₂O (290 mg/L) which was modified from Warner et al. (1985) and Sieverding (1991). Solution-II included (NH₄)₂SO₄ (19 mg/L) as a N-source adding insoluble Ca₃PO₄ (100 mg/L) as a P-source and solid CaSO₄.2H₂O (290 mg/L) which was modified from Elems and Mosse (1984) and Sieverding (1991). Solution-III was comprised of one quarter Hoagland solution and insoluble Ca₃PO₄ (100 mg/L) (modified from Wang and Tschén, 1994).

Solution-IV was made from full-strength Hoagland solution with low P (0.62 mg/L) (Millner and Kitt, 1992).

Mycorrhizal root colonization

Root samples were washed with tap water to remove sand substrate. The roots were then immersed overnight in a 2.5% KOH solution. The KOH solution was washed off and the roots were rinsed three times with distilled water. The roots were acidified using 1% HCl (Biermann and Lindermann, 1981), stained with trypan blue and mounted on slides using polyvinyl alcohol lactic acid glycerine (PVLG) for viewing under a compound microscope. Percentage of root length with internal AM fungal infection was determined (Brundrett et al., 1996).

Mycorrhizal spore collection

Spores from the rooting medium were extracted by wet sieving with a 125 µm and 850 µm sieve set. The spores retained on 125 µm to 500 µm sieves were collected in a beaker and recovered by sucrose density centrifugation (Danny and Brenda, 2006) and spores from the upper layers were washed on a 45 µm sieve, then suspended again in distilled water. Aliquots of the spore suspensions were distributed onto a filter paper for counting with a stereomicroscope (Simpson and Daft, 1990). Counts were made from five replicates to determine spore per milliliter, and then multiplied by the total volume of the spore suspension to determine total spore count. Spore populations were expressed per gram of oven-dry sand after sample moisture was determined (Millner and Kitt, 1992).

Experimental designs

Effectiveness of AM fungal infection on roots of leaf lettuce varieties

The experimental design was a completely randomized design (CRD) with five replications. There were four leaf lettuce cultivars; green leaf, red leaf, Granada lettuce and oak leaf. In the control, the plants were not inoculated with AM fungi. One seedling was planted directly into 30 mL pot filled with sterilized moistened fine sand (Gaur and Adholeya, 2000) and inoculated with 50 spores of *G. verruculosum* (Ahmad and Raddad, 1995). The plants were fed weekly with 40 mL of a quarter-modified Hoagland solution (Simpson and Daft, 1990). After four weeks, AM fungal infection of roots was determined.

Growth and AM root colonization determination of red leaf lettuce when cultured in modified half strength of Long Ashton nutrient solution (LANS) versus modified Hoagland nutrient solution (HNS)

A completely randomized design (CRD) factorial experiment was conducted. Five replications of two nutrient solutions which were inoculated and non-inoculated with AM fungi were evaluated. The plants were initially watered and supplied with approximately 15 mL each of LANS solution or HNS solution at five-intervals. In the control, seedlings were sown under similar conditions but without AM fungal inoculate. After four weeks, the roots were examined to determine the AM fungal infection. The AM infected seedlings were then transferred into the hydroponics to be cultivated for another four weeks. In order to transplant the

cultivars, the pots were submerged in five-liter hydroponic containers (five plants per container) containing either LANS or HNS medium. The nutrient solutions were changed weekly; pH and electrical conductivity (EC) were adjusted daily. Aeration of the nutrient solutions was supplied via an air pump producing a fine stream of bubbles through aquarium stones. At harvest time, dry plant shoots and roots were weighed and percentages of root colonization were determined.

The impact of four modified Hoagland nutrient solutions (Solution-I to Solution-IV) on lettuce-arbuscular mycorrhizal growth, root colonization and AM spore production

The experimental design was a completely randomized design (CRD). There were four plant nutrient solutions (solution-I, solution-II, solution-III and solution-IV). Five replications of healthy seedlings, which had been colonized by AM fungi, were transplanted into the hydroponic system. At four weeks, the shoots were severed just above the crown. Plant root samples were taken for evaluation of mycorrhizal colonization. Dry weight of shoots, roots and the amount of AM spores from the root substrate were determined.

Statistical analyses

Data were analyzed by analysis of variance (ANOVA) using a completely randomized design. The differences between the treatments were examined by Fishers least significant difference (LSD) test at a significance level of 95%.

RESULTS AND DISCUSSION

Effectiveness of AM fungal infection on roots of leaf lettuce varieties

In the first experiment, all the varieties of lettuce used in this study were subjected to mycorrhizal infection (Figure 1). The percentage of colonization varied greatly among different plant varieties. There was no root infection in non-mycorrhizal plants. The maximum root infection was recorded in red leaf (81.49%) which was not significantly different from the green leaf (78.15%), followed by granada leaf (32.85%) and oak leaf (23.47%), respectively.

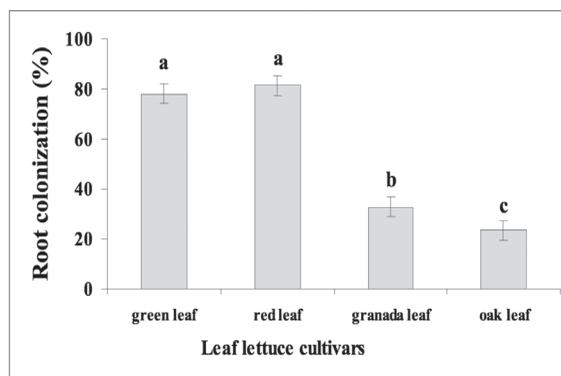


Figure 1. Percentage of root colonization in four leaf lettuce varieties (green leaf, red leaf, granada leaf and oak leaf) with *G. verruculosum*.

Leaf lettuce (*Lactuca sativa var. crispata* L.) is a horticultural crop that was highly colonized by AM fungi. Root colonization depended on types of host plant. The red leaf has more colonization than the other varieties. This indicated that AM fungi are specific to the type of host plant and that there is a variation in mycorrhizal infection among commercial lettuce cultivars (Bever, 2002). This study supports Jackson et al., (2002) who reported that mean colonization ranged from 14 to 31% with highest colonization in Romaine lettuce, followed by crithead, red leaf and green leaf, respectively. There is evidence of specific differences in the fungal response to the host plant that is consistent with those of other reseachers (Van der Heijden et al., 1998; Bever, 2002).

Growth and AM root colonization determination of red leaf lettuce when cultured in modified half strength of Long Ashton nutrient solution (LANS) versus modified Hoagland nutrient solution (HNS)

Long Ashton nutrient solution (LANS) and Hoagland nutrient solution (HNS), which are widely used as media for hydroponics, were examined for their effects on lettuce growth and root colonization. The initial AM root colonization of red leaf lettuce seedlings was approximately 80%. From the result in Table 1, dry weights of shoot and root for non-mycorrhizal (NAM) samples were not significantly different from those of mycorrhizal (AM) samples in the same media. However, in the comparison between two different media, the HNS medium seemed to have higher potential to enhance the growth of shoots (7 g.) and roots (2 g.) than LANS medium (0.2 g. of shoots and 0.03 g. of roots). No colonization was observed in NAM samples, while 47.04% and 7.09% of root colonization was observed in AM samples grown in LANS and HNS media, respectively. This result indicated that the HNS medium was better for lettuce production. Further, chlorosis was observed in lettuce grown in LANS medium while the plants in HNS culture had a normal green color. Since the total nutrient concentration (indicated by EC) of HNS (0.7 dS/m) was seven times greater than that of LANS medium (0.1 dS/m) and the available phosphorus (P) concentration in the HNS medium was twice that of the P concentration in LANS medium, the mineral concentration of HNS appeared to be enough for hydroponic lettuce production.

Table 1. Effects of LANS and HNS media on plant growth of non-mycorrhizal (NAM) and mycorrhizal (AM) lettuce and root colonization percentage.

Parameters	LANS		HNS	
	NAM	AM	NAM	AM
Shoot dry weight (g)	0.16 b*	0.19 b	7.22 a	7.45 a
Root dry weight (g)	0.03 b	0.03 b	1.66 a	1.74 a
Total root colonization (%)	0.00 c	7.90 b	0.00 c	47.04 a

*Means followed by the same letter in the same row are not significantly different by ANOVA and the least significant difference (LSD) test (P = 0.05).

A hydroponic culture is one method for producing AM inoculum in order to enhance commercially produce mycorrhizal horticultural crops. LANS medium is used in the culture of linseed, sorghum and wheat while HNS medium is used in the culture of linseed (Hawkins and George, 1997), lettuce (Sieverding, 1991), maize, beans (Ellems and Mosse, 1984), lucerne, clover and capsicum (Mosse and Thompson, 1979). The total nutrient concentration (indicated by EC) of HNS was greater than that of the LANS medium. No significant difference in average initial root colonization between treatments was observed when mycorrhiza was grown in sand substrate. This indicates that all nutrient media used in this study were suitable to mycorrhizal infection for the tested plants. The shoot and root growth is generally an important parameter when evaluating responses in nutrient concentration associations. Lettuce, a fast growing crop requiring high amount of water and nutrients demand for growth, had demonstrated chlorosis when grown in LANS medium. On the other hand, plants in HNS culture were of a normal green color, showing that nutrient concentration supplied was sufficient for crop requirements. The AM fungi maintained colonization in plants grown hydroponically using HNS. These results are in contrast with those of Hawkins and George (1997) who reported that *G. mosseae* maintained a colonization of 66% in wheat plants grown in hydroponics using LANS but much lower colonization when HNS was used. The poor colonization of lettuce using LANS under these experimental conditions was due to the relatively deficient nutrient concentration, which inhibited plant growth and root colonization. Thus, the HNS medium was developed to optimize the growth of lettuce-mycorrhizal plants in a hydroponic system.

The impact of four modified Hoagland nutrient solutions on lettuce-arbuscular mycorrhizal growth, root colonization and AM spore production

Only mycorrhizal plants were applied in different solutions (Table 2). Although the result of the previous experiment showed that HNS was supportive for mycorrhizal spore propagation in the lettuce host, further understanding of suitable mineral concentration is needed to achieve the highest spore production of *G. verruculosum* on a lettuce host. In this experiment, electrical conductivity (EC) was measured as the indicator of total nutrient concentrations in the media. As seen in Table 2, the highest EC concentration was recorded in Solution IV (2.30 dS/m), followed by Solution-III (0.70 dS/m), Solution-II (0.50 dS/m) and

Solution-I (0.50 dS/m), respectively. Lettuce growth, root colonization and spore population were measured after 4 weeks of transplanting. The highest shoot and root dry biomass was found in Solution IV, followed by Solution-III. Fewer responses on lettuce growth were found in Solution-II and Solution-I. In contrast to the total root colonization (86.3%) and spore production (303 spores/g. dry sand) became highest whereas the lowest production (41.5% and 32 spores/g. dry sand) was found in Solution IV. The significant difference ($p < 0.05$) in root colonization and spore population between Solution-I and Solution-II indicated that the fungi preferred NO_3^- to NH_4^+ as an N source. In Solution-I and Solution-II media of which insoluble Ca_3PO_4 and solid $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ were included, the lettuce significantly exhibited positive stimulation in terms of root colonization and spore production, compared to that in Solution-III and Solution-IV. This finding suggested that the type of nutrient solution affected the AM fungi performance.

Table 2. Effects of four different modified Hoagland solutions (Solution-I, II, III and IV) on shoot and root dry weight, total root colonization percentage and spore production of mycorrhizal lettuce plants after culturing in a hydroponic system.

Parameters	Solution-I	Solution-II	Solution-III	Solution-IV
EC (mS/cm)	0.50 b*	0.50 b	0.70 b	2.30 a
Shoot dry weight.(g)	1.18 c	1.86 c	8.09 b	15.50 a
Root dry weight.(g)	0.36 c	0.61 c	2.03 b	4.18 a
Root colonization (%)	86.29 a	71.94 b	46.20 c	41.50 c
Spore number /g. dry sand	303.0 a	160.0 b	47.0 c	32.0 c

*Means followed by different letters in the same row are significantly different according to least significant difference (LSD) test ($P = 0.05$).

Application of the Hoagland solution with insoluble-P enhanced both spore production and infectious AM fungal propagules (Gaur and Adholeya, 2000). Douds and Schenck (1990) also demonstrated that low levels of P help maintain high levels of AM infectious. High concentrations of other nutrients (except N, P and Ca) increase the amount of photosynthate available for colonization and sporulation. The microelement compositions of all media were similar to that of the Hoagland solution which is generally suitable to use (Mosse and Thompson, 1984). The total nutrient concentration showed a maximum concentration in solution-IV, followed by solution-III, solution-II and solution-I, respectively. In the present study, solution-IV of the media that had the highest nutrient concentration produced a large increase in shoot and root weight. In contrast, the total root colonization and spore production were significantly different between treatments and they decreased when grown in high nutrient concentration. The poor sporulation was attributed to the full strength Hoagland solution. In the system based on a soluble-P source, pH and nitrogen source are probably less critical, although too much inorganic nitrogen can depress or inhibit mycorrhizal infection (Elems and Mosse, 1984). This suggests that for the mycorrhizal-leaf lettuce with insoluble-P,

the solution containing NO_3^- as an N-source was better than that containing NH_4^+ as an N-source. This contrasts with the report of Mosse and Thompson (1979) that mycorrhizal-head lettuce preferred NH_4^+ -N. This indicated that inorganic-N form of the salts used is also suitably selected in relation to plant-mycorrhizal system because both plant and AM fungi growth have to be balanced and they have different optimum requirements. This also supports the concept that the ideal nutrient solution for AM inoculum production will be low in plant nutrient concentration. The modified Hoagland's solution plus Ca_3PO_4 resulted in spore production ranging from 160 to 303 spores/g. dry sand, which was six times greater than commercial inoculum (Evans and Snow, 1999; Tarbel and Koske, 2007). This result is consistent with the finding of Millner and Kitt (1992) who found that spore production in the soilless system exceeded of traditional soil culture by 32-362%. Sporulation was more closely related to infected roots than to any other parameter, confirming the results of other researchers (Simpson and Daft, 1990). Our study supports Bhowmik and Singh (2004) who reported that high root colonization also improved the arbuscular mycorrhizal spore number, since these two phenomena are often closely correlated (Jarstfer et al., 1998). A negative correlation between spore production and shoot and root fresh weight was obtained. In addition, electrical conductivity of nutrient media was negatively correlated with spore production because it depressed or inhibited mycorrhizal colonization (Mosse and Thompson 1979; Elems and Mosse 1984). In the modified HNS medium containing NO_3^- and insoluble Ca_3PO_4 , the AM fungi infected and developed well with red leaf lettuce and large amounts of fungal spores were produced. This offers the potential of determining nutrient solution directly in fungal hyphae through plant-fungus interaction.

However, further study should be conducted to test the inoculum potential of these hyphae by most probable number (MPN) (Lee et al., 1996; Mohammad et al., 2000) or number of infectious propagules (IP) (Gaur and Adholeya 2000; Gaur and Adholeya 2002) compared with those of other culture systems so as to be qualified as commercial inoculum. Moreover, relatively constant spore densities during the vegetative phase and a sharp increase at the reproductive stage have been reported for various crops (Sutton and Barron, 1972; Saif and Khan, 1975; Pozzebon et al., 1992). It is likely that senescence and dead roots stimulate the onset of sporulation at the end of the host's growing season as observed by others researchers (Simpson and Daft, 1990; Ahmad and Raddad, 1995). The suitability of harvest date for growing lettuce-mycorrhizal fungus must be tested in further experiments.

CONCLUSION

The root colonization depended on the lettuce varieties. The AM fungal infection was the greatest in red leaf lettuce. Two nutrient media, modified Long Ashton (LANS) and modified Hoagland medium (HNS), were compared for culturing *G. verruculosum* on red leaf. The total root colonization was shown to be greater in HNS compared with that in LANS medium. Thus, the HNS medium

was developed for sporulation of AM fungus. In hydroponic culture, the modified HNS medium containing NO_3^- plus insoluble Ca_3PO_4 and solid $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ was the most appropriate solution because it produced the highest number of AM spores.

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