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Full Length Research Paper

An evaluation of large scale production of AMF inoculum through root organ culture

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The Arbuscular Mycorrhizal Fungus (AMF) plays a symbiotic vital role in most of the plant species by maintaining health, nutrient availability, against water stress, heavy metal resistance, against dreadful diseases with higher yields. To reach the requirement of AMF, large scale production practices can be required to produce specific strain aseptic inoculum. Carrot roots were selected for mass production of mycorrhizal spores among tomato, potato, sweet potato, soybean, amaranths and carrots through root organ culturing method. Aggregated roots were cultured and developed as root clumps for continuous sub-culturing. With slight modification of root organ culture method and split plate method, in containers shown significant increase in the extra radical spore count. Maximum *Rhizophagus irregularis* spore count was achieved with molecular weight (MW) medium among three different media MW medium, M medium and MSR medium. Almost 99204±1438.10 *R. irregularis* propagules were achieved with sugar free MW medium per container in 90 days of incubation in dark. M medium fortified with 1% sugars yields 16236±1186.70 intraradical and 39458±1098.00 extra radical spores were observed. To achieve continuous production of Mycorrhizal inoculum, transformed callus root clumps were infected with *R. irregularis*. This AMF inoculum developed in the sugar free medium is less contagious and highly productive during the process of harvesting, packing, transportation and preserved for longer duration without much contaminants.

Key words: Arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, root organ culture, endomycorrhizae.

INTRODUCTION

The arbuscular mycorrhizal fungus plays crucial role to attain plant health and soil fertility. The plant root

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association with the mycorrhizal fungi overcome the challenges like water stress (Liu et al., 2015; Bompadre et al., 2014), heavy metal resistance against aluminium and lead in *in vitro* (Gavito et al., 2014) chromium immobilization (Wu et al., 2016), mercury contaminated soils, copper stress (Almeida-Rodríguez et al., 2015; Ambrosini et al., 2015), arsenate and arsenite stressed soils (de Andrade et al., 2015; Cattani et al., 2015), against oxidative stress (Driai et al., 2015). In association with soil microbial flora AMF supports plant systems on nutrient availability (Tekaya et al., 2016), bioremediation (Ingrid et al., 2016; Fester, 2013) and heavy metal resistance (Dhawi et al., 2016). AMF association also gives resistance against many diseases like black sigatoka in *in vitro* banana (Anda et al., 2015) and various soil borne plant pathogens (Tahat et al., 2010). In combination of other beneficial microbes AMF can also show the yield variation like with *Trichoderma harizianum* in potato (Buysens et al., 2016; Moreira et al., 2016; Hijri, 2016).

In sustainable agriculture, AMF has great potential in crop production and environment preservation (Oruru and Njeru, 2016; Aggarwal et al., 2011). Unfortunate dependency on toxic chemical pesticides and synthetic fertilizers in farming practices leads to the degradation of soil health, water pollution, natural pollution and ultimately human and animal health (Aktar et al., 2009). By using the AMF in farming as biofertilizer, can minimize the usage of toxic chemical pesticides and synthetic fertilizers (Oruru and Njeru, 2016). In recent research findings, *Rhizophagus irregularis* plays key role in reclamation of saline soils and heavy metal contaminated soils (Lenoir et al., 2016; Lone et al., 2008). Natural presence of these AMF in soils was disappeared, with the continuous application of these synthetic chemicals and deep tillage (Berruti et al., 2014). For sustainable agriculture, it is mandate to re-establish the mycorrhizal population in soils. But for farmers it is very expensive, to get uncontaminated pure mycorrhizal inoculum because of its obligate symbiotic nature (Ceballos et al., 2013).

To achieve sustainable agriculture system, it is essential to re-establishing the mycorrhizal populations in the soil for uplifting the soil health, plant growth and yield (Wahbi et al., 2015; Ceballos et al., 2013). But because of its obligate symbiotic nature of reproducibility and duration of its life cycle, makes major challenge for large scale production (Berruti et al., 2016). High levels of phosphorous and sugars are also major limiting factors to develop the AMF inoculum (Habibzadeh, 2015; St-Arnaud et al., 1996).

The development of arbuscular mycorrhizal fungus (AMF) in *in vitro* root organ cultures has greatly influenced by the potential for research and large scale production of uncontaminated inoculum production. *In vitro* root organ culture system was first reported by White (1943). AM fungal culture development under *in vitro* root organ culture process is the most promising way to obtain good amount of extraradical spores in a short span of time with contamination free inoculum (Binondo et al., 2012). The excised roots on synthetic mineral media supplemented with vitamins and a sugar source profuse root proliferation, characterized by the formation of numerous braches with small hairy roots with few plant species. The formation of hairy roots is essential for the development of vigorous root biomass and the establishment of continuous AMF cultures. In plant growth regulators, cytokinins, gibberellins (Takeda et al., 2015) may influence the AMF colonization and symbiotic association. Mosse and Hepper (1975) were first developed this root organ culture system by using *Lycopersicum esculentum* Mill.

AMF cultivation techniques based on *Agrobacterium rhizogenes* transformed roots provide the development of the AMF inoculum *in vitro* (Bécard and Fortin, 1988; Adholeya et al., 2005; Ijdo et al., 2011; Schultze, 2013). After the development of monoxenic cultures of AM fungus through root organ culture made possible for the continuous observations of fungal colonization and mycelium development as well as the sporulation. Minimal mineral media (M) and modified Strullu-Romand (MSR) media were successfully used for AM fungal colonization on non-transformed tomato root cultures (Diop et al., 1994a; Bago et al., 1996). These root organ culture studies giving better knowledge in mycelium development (Bago et al., 1998a), functional aspects of the mycorrhizal root symbiosis (Debiane et al., 2009), dynamics of sporulation in *in vitro* host roots (Declerck et al., 2001; Declerck et al., 2004; Voets et al., 2009; Ijdo et al., 2011), AMF spore ontogeny (De souza et al., 2005), study of species level reproduction cycles and nutritional requirements (Labidi et al., 2011; Ijdo et al., 2011) and large scale production of viable contamination free inoculum (Tiwari and Adholeya, 2002; Voets et al., 2009; Ijdo et al., 2011) with *Solanum tuberosum* (Puri and Adholeya, 2013).

Only a few species of AMF strains have been successfully established *in vitro* through root organ culture technique (Tiwari and Adholeya, 2002; Ijdo et al., 2011). Although having the potential use of the *in vitro* root organ cultures, have obvious limitations in many aspects the AM symbiosis. Importantly the plant host is replaced by a genetically transformed root organ, affected by the absence of regular photosynthetic organs and normal hormonal effects. Transformed root organs may not be active for too many subcultures for continuous production. Sucrose is added to the culture medium to compensate photosynthesis were effect the root-fungus interface. Sugars at this interface may modify the biochemistry of the plant fungal interaction. According to Fortin et al. (2002) these sugar concentrations might affect the arbuscules and vesicles development in *in vitro* transformed carrot roots. This study mainly focuses on selection of media and host for large scale production of AMF inoculum through root organ culture and also to

increase the productivity of AMF inoculum.

MATERIALS AND METHODS

For *in vitro* mass production of endomycorrhizae, actively growing host was required. For getting Ri T-DNA transformed roots in *in vitro*, virulent (*Agrobacterium rhizogenes*) strain was required for the transformation (Mugnier and Mosse, 1987).

Bacterial strain propagation

Agrobacterium rhizogenes slant culture MTCC-532 was bought from the Microbial Type Culture Collection (MTCC), Chandigarh, India. A bacterial suspension of this strain was prepared and inoculated into various media like Lauria-Bertani (LB) broth, yeast mannitol broth (YMB), nutrient broth and yeast extract peptone (YEP) medium incubating overnight on a incubating shaker (ACMAS, New Delhi, India) at 28±2˚C at 150 rpm. Almost all media gave good growth but YMB medium showed excellent turbidity at pH 7.0 (0.739±0.004 O.D at 600 nm (U.V spectrophotometer, ELICO, India)). YMB medium (1.0 g/L yeast extract, 2 g/L mannitol, 3 g/L glucose, 5 g/L sucrose, 0.5 g/L K_2HPO_4, 0.1 g/L NaCl, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L NH_4Cl, 0.05 g/L $CaSO_4.2H_2O$) was used for further experiments for subsequent sub-culturing of *Agrobacterium rhizogenes* in every month. *A.rhizogenes* cultured in 250 ml Erlenmeyer flask and incubated at in darkness on rotary shaker at 150 rpm.

Carrot hairy root culture

Carrot roots were found that the easiest source to transform and regenerate among Tomato hypocotyles regions (*Solanum lycopersicum*), Amaranthus hypocotyle regions (*Amaranthus caudatus*), Carrot horizontal root slices (*Daucus carota* sub sps. *sativus*), Potato discs (*Solanum tuberosum*) (Puri and adholeya, 2013), Sweet potato root slices (*Ipomea babatas*) and Soybean cotyledons (*Glycine max*). Freshly harvested (greater than 1 inch width) healthy carrots from polyhouse were taken for easy transformation with *Agrobacterium rhizogenes*. Carrots were washed thoroughly with tap water and surface sterilised in 1 % sodium hypochlorite for 15 min (Bécard and Fortin, 1988), 30% H_2O_2 for 5 min, 0.1 % $HgCl_2$ for 10 min with continuous stirring and further rinsed three times (5 min each) in sterile distilled water. Prior to cutting into discs they were dipped in 70% ethanol and flamed for 3 to 4 s. Then the carrots were sliced into 4 to 6 mm thick discs and placed on the MS (Murashige and Skoog, 1962) medium, basal sides faced upright position.

Transformation of carrots (*D. carota* L.)

A. rhizogenes (24-48 h old) in YMB broth was directly used for inoculation of carrot root slices. A loop full of suspension was applied on the surface of carrot root discs aseptically in petri-dishes containing MS medium with 3% sugars and incubated at 26±2ºC in dark. After 3 to 4 weeks of incubation, callus and roots were initiated from the cambium (more than 6 cm length) were excised and placed on freshly prepared MS medium plates. Healthy white root tips with profuse lateral roots showing negative geotropism on the surface of synthetic MS medium. To get *Agrobacterium* free transformed carrot roots, weekly sub-culturings were done on MS medium plates with antibiotic carbenicillin 500 mg/L for 3 to 4 weeks with decreasing concentrations of antibiotic (400, 300, 200, 100 mg/l). The antibiotics like carbenicillin, cefotoxime (250 mg/l) and gentamycin (100 mg/l) were used to control the bacterial growth in transformed hairy roots. To establish bacterial free clone culture 3 to 4 subsequent subcultures were required.

Development of root clumps and maintenance

Subsequently sub-cultured transformed carrot hairy roots in M-medium were gradually lost their regeneration capability for continuous development of hairy roots in the fresh medium also. To get the fresh transformed roots all the time was difficult and time consuming. To overcome this continuously reproducing host must be available all the time. For this, these carrot root clumps were developed in MS medium. After prolonged incubation of transformed roots in media, some of the root tips were shown 3 to 4 sub roots. The aggregates of root cells were carefully collected from the 90 days old containers and inoculated into the fresh MS medium with 3% of sucrose and incubated in dark at 26 ± 2˚C (BOD incubator, Bio Technics India, India). After 45 to 60 days of incubation, the fully grown callus root clumps were sectioned and were used for the multiplication of several root clumps in MS medium. The ~ 1 cm sections of these callus root clumps were gave multiples of actively growing white hairy roots in fresh M-medium. To get more callus root clumps for large scale production, sections of clumps were further sub-cultured into the MS medium with 3% sucrose. These callus root clumps with hairy roots were used as regenerative hosts for the endomycorrhizal infection and mass production of mycorrhizal spores.

Spores inoculation and culture development in petri plates

Inoculum of *Rhizophagu sirregularis* was isolated from the Narsapur Forest, Telangana, India, identified and developed in the Rand D Centre for Conservation Biology and Plant Biotechnology, Shivashakti Biotechnologies Limited, India. The *R. irregularis* spores (8-10) were inoculated into a Petri dish with actively growing transformed hairy root clumps in M- medium and incubated in BOD incubator. The inoculated petri plates were incubated at 26±2ºC in dark and monitored daily under compound microscope for spore germination and root infection.

Optimization of mass production in containers

For large scale production of mycorrhizal spores, 250 ml containers were used for providing the more nutrients and to get the more extraradical spores. Proliferating callus root clumps were explanted in to individual container containing 100 ml of three different media like MW medium, M medium and MSR medium with 1% sucrose concentration in containers and incubated for 90 days 26±2ºC in dark growth rooms. The spores formed in the medium were counted and root colonization by *R. irregularis* was assessed by magnified grid line intersection method (McGonigle et al., 1990) after roots were stained. The whole root length from each container was examined to calculate the intraradical structures of AMF.

Modified split plate method in containers

Bi-compartment plate system was introduced by St-Arnaud et al. (1996), to study the impact of sucrose on spore and achieved 10 fold increased spore count. By implementing this method, for mass production of AMF in 250 ml containers, filled with 100 ml of M medium without sucrose solidified with 0.4% CleriGel (Phytagel in Himedia, India) and autoclaved for sterilization. After solidification of M medium in the containers, it was inoculated with mycorrhizal infected active root clump and partially immersed with 20 ml of M medium made up with 1% agar and sucrose (1%-C1, 2%-C2 and

3%-C3 concentrations) at 40±2°C. The prepared bottles were incubated at 26±2°C in dark for 90 days. Modified split plate method was also tested with 3% sucrose concentration with different media MW medium, M medium and MSR medium in containers and incubated at 26±2°C.

After 90 days of incubation in dark the spores were assessed after deionization of phytagel with 10 mM citrate buffer. To count extraradical spores formed outside of the root were counted under stereo zoom microscope (Olympus SZ61 TR, Japan) and intraradical spores formed inside of the root were stained and counted under compound microscope (Olympus CX41 RF, Japan). GraphPadInStat 3.10 version was used for the statistical analysis.

RESULTS

Cultivation of transformed roots

Sections of carrot roots were provided as the potential donor explants for Ri T-DNA mediated transformation with *A. rhizogenes* strain MTCC-532 in YMB medium. YMB medium was selected out of the LB medium, nutrient medium and YEP medium based on the optical density at 600 nm. Among different media YMB medium was got 0.739 as highest optical density within 24 h of incubation in dark at 28±2°C and 150 rpm. Among Tomato (40% of hypocotyles), Amarathus (10% of hypocotyles), carrot (70% of root slices), Potato (40% of potato discs), Sweet potato (30% of discs) and soybean (40% of cotyledons), carrots were transformed easily and shown maximum percentage of transformation. After 7 days of inoculation, successful transformation was shown by the maximum callus formation at cambial regions and direct emergence of roots from endodermal sites.

70% of root slices were shown the callus formation by placing the basal sides of the slices in upright position on the MS medium. Some of the carrot root discs were rotten and became brown due to over growth of *A. rhizogenes*. The full-fledged transformed roots were mostly emerged from endodermal regions of the carrot sections after 3 to 4 weeks period (Figure 1A). With the exposure of cambium in carrot root discs, the hairy root initiation made easy to generate healthy hairy roots. The vigorously grown thicker roots with more lateral roots, greater than 5 cm size were cut off and transferred to MS medium with 3% sugar and specific antibiotics to get bacterial free roots. Carbenicillin and cefotoxime were successfully found to control the *Agrobacterium* growth in transformed hairy roots. These *Agrobacterium*-free roots were vigorously grown in hormone free medium with negative geotropism (Figure 1B).

Development of callus root clumps

The continuously sub cultured transformed carrot roots were gradually lost their reproducibility. These roots became brown and inactive to reproduce. These aggregates placed in the fresh MS medium gave the opaque, massive callus root clumps within 45 to 60 days (Figure 1C) with multiple fresh hairy roots. The 1 cm sections of these callus root clumps again in fresh MS medium with 3% sucrose were giving the massive callus root clumps in subsequent subculturings also (Figure 1D). These callus root clumps in M medium produced the actively growing white hairy roots vigorously (Figure 1E). These root clumps were also found inactivated slowly in the fresh M medium with continuous subcultures. But it was found that, ~ 1 cm callus root clumps were very active in the MS medium (3% sucrose) with number of subcultures. By maintaining in MS medium root clumps were maintained during the entire cycle of production.

Inoculation and development of mycorrhizal inoculum

The spores of *R. irregularis* was successfully germinated and colonized the hairy roots within a week (Figure 1F). The monoxenic spores were germinated within 3 to 4 days on M medium with subtending hyphae and spreaded all the directions randomly (Figure 1G). Within 2 to 3 days hyphae contacted the extensively grown hairy roots from callus root clumps. Hyphae proliferated vigorously on the surface of roots forming various shapes of appressoria. Extra radical spores were started developing after 30 days, with incubation in 26±2°C temperature in BOD incubator. In prolonged incubation (4 months) sporulation was randomly increased and roots turned orange to brown colour. But 3 to 4% of monoxenically developed spores were aborted by leakage and spilled around the medium (Figure 1H). With prolonged incubation, roots were turned brown due to shortage of nutrients and root exudates (Figure 1I). Establishment of AMF in petri plates were supported the observation of mycelial growth, spore formation and hyphal network. But for mass multiplication of extraradical and intraradical spores of *R. irregularis*, containers (bottles or jars) were opted.

Large scale production in containers

After 5 days of inoculation in 100 ml containers, mycorrhizal hyphae were spreaded into the medium and new white hairy roots were developed from the callus root clump. By increased growth of transformed hairy roots from the callus root clump, density of mycelial network was increased in the medium. Because of negative geotropism in transformed carrot roots, hairy roots were mostly developed on the surface of medium by hosting the AMF. In 60 to 90 days time period, sporulation was vigorously developed in the containers (Figure 2A). The density of mycelial threads and spores was very high near roots and diluted at the bottom of the container. After 90 days of incubation, the roots were separated

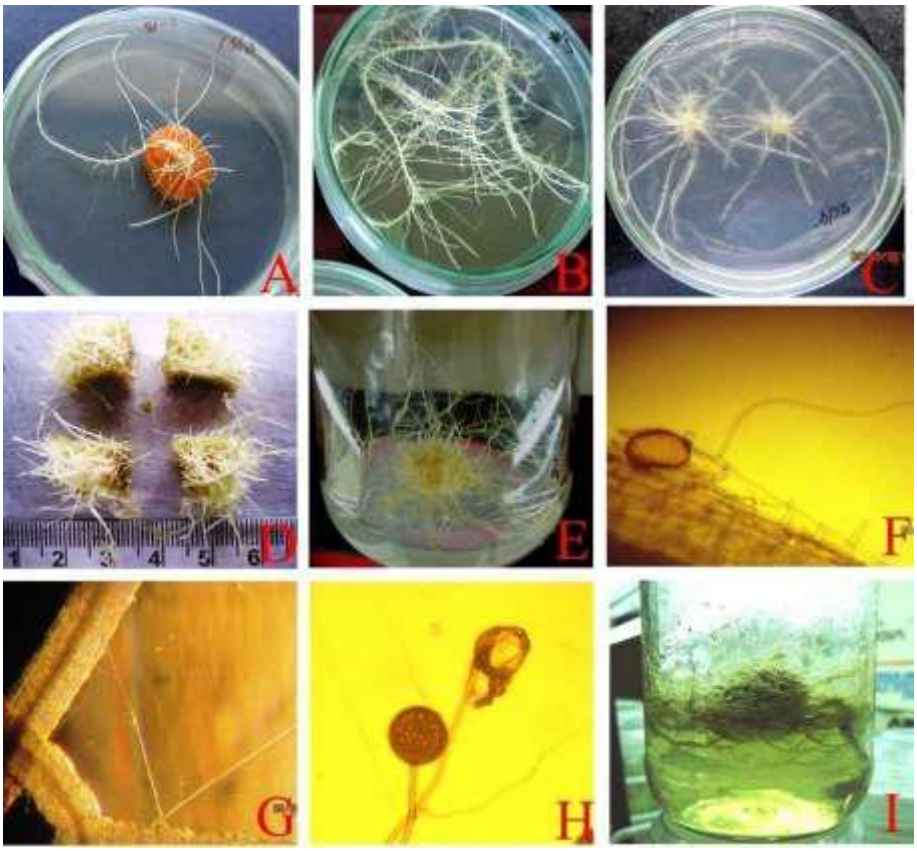

Figure 1. Large scaleproduction of *Rhizophagus irregularis* in containers (A) Transformation of carrot roots in MS medium; (B) Sterilized transformed root bits with lateral roots in MS medium; (C) Transformed callus root clumps on MW medium; (D) Large scale production in containers with M medium; (E) Modified split plate method in container with MSR medium; (F) Magnified view of extra radical spores developed in containers with MW medium; (G) *In vitro*grown *R. irregularis* spore developmentin M medium; (H) Intraradicalspores in transformed hairy roots; (I) Sieved extra radical spores under stereo microscope.

from the container and phytagel based medium was de-ionized with (10 mM) citrate buffer. The medium was sieved with mesh size 300, 150 and 60 standard test sieves and spores counted under stereomicroscope with gridline intersect method (Figure 2B).

Roots were extensively grown in all the three media and shown almost similar percentage of root colonization as 88% (Figure 3). The ~1 cm chopped and stained root bits were made possible to count intra radical spores. The intra radical spores were slightly higher in MW medium (21077±2096.3) than in the MSR medium (19639±752.4) but significantly varied from M medium (16236±1186.7) (Figure 2C). The extraradical spore count was significantly higher in M medium (39458±1098.0) than the other two media, MW medium
(21077±2096.3) and MSR medium (19639±752.4) (Figure 2). M medium was supported to develop good number of *R. irregularis* spores than MW medium and MSR medium with 1% of sucrose concentration.

Implementation of split plate method in containers

By the implementation of split plate method in containers, within 10 days of incubation partially immersed root clumps were developed several new white hairy roots with extended lateral roots in 3% sucrose concentrated bottles than 2 and 1% concentrations. The extended hyphae were developed in the all the three media, but extensive mycelial network was developed in the containers, having 3% of sucrose than the other concentrations (Figure 2E). But the sporulation was developed slowly 40 days onwards in bottom phase (M medium without sucrose). After 40 days of incubation, establishment of spores was randomly increased.

The de-ionised and sieved M medium was filled with 88271±965.14 total spores in C3, 76374±758.49 in C2 and 40665±1730.4 in C1 respectively. The percentage of root colonization was significantly decreased with the increase of sucrose concentration (Figure 5). C3

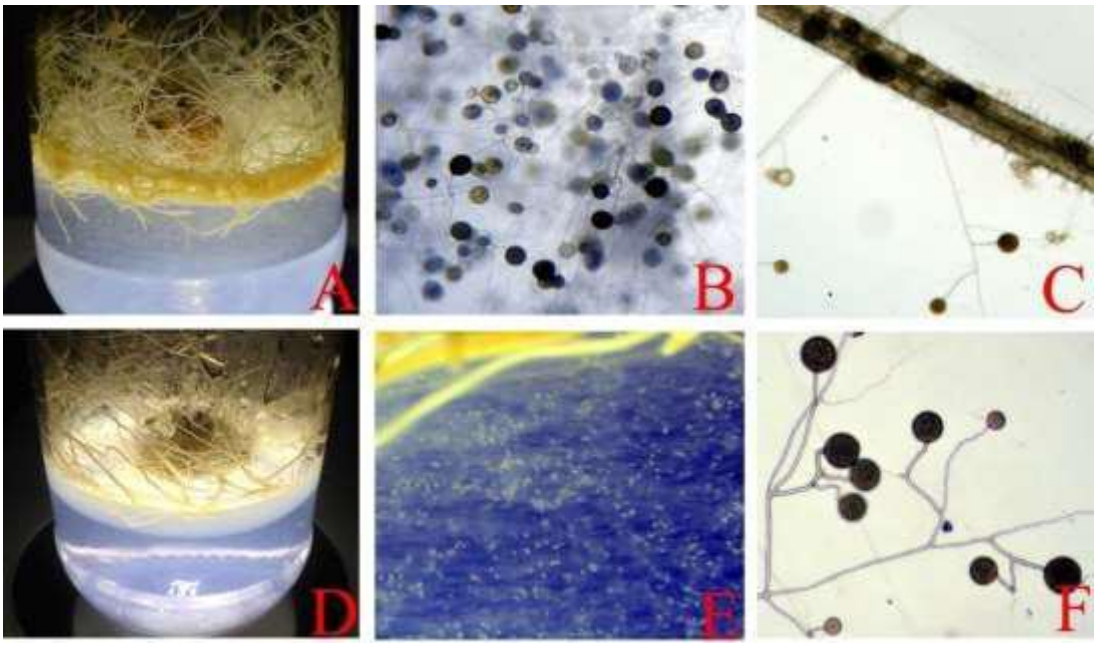

Figure 2. (A) Large scale production in containers with M medium B) Sieved extraradical spores under stereo microscope (C) Intraradical spores in transformed hairy root (DModified split plate method in container with MSR medium (E) Magnified view of extra radical spores developed in containers with MW medium (F) In vitro grown Rhizophagus irreglaris well developed spore in M medium.

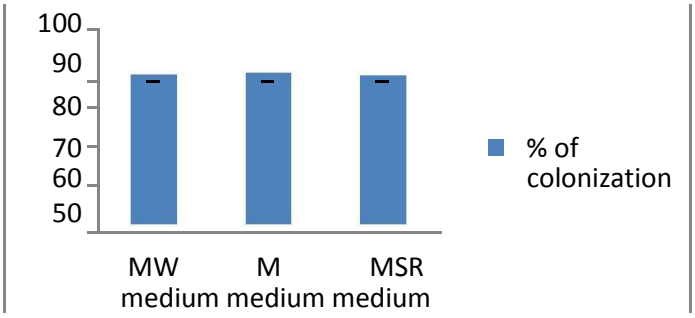

Figure 3. Percentage of colonization in containers with three different media.

(72329±664.00) containers were shown highest extraradical spore count than the C2 (59023±875.27) and C1 (22941±1244.6). The intraradical spore count was observed almost same in C2 (17351±720.73) and C1 (17724±1044.0) but lowest intra radical spore count was recorded in C3 (15972±679.66) (Figure 4). The entrapped colonized callus root clumps with different sugar concentrations were gave significant variations in the development of *R. irregularis* spores and development of fresh hairy roots from the clumps.

During the incubation, C3 containers were responded early with fresh hairy roots from the callus root clump and showed the hyphal development towards sugar-free M medium within 2 to 3 days. Other media (C2 and C1) were also simultaneously showed the positive results with the difference of 7 to 10 days. Within 20 days mycelial density was increased tremendously in C3 M medium. In 90 days of incubation massive sporulation was observed in the sugar-free phase of C3 (Figure 2F) solidified with phytagel and good regenerative root clumps were developed in the sugar containing C3 M medium solidified with agar.

The comparison study with three different media with modified split plate method, MW medium was shown significant higher spore production (99204±1438.1) than the M medium (89514±2272.1) and MSR medium (82142±1059.3). The extraradical spore development was very high in sugar free MW medium (82067±1343.3) than M medium (72886±1440.3) and MSR medium (63501±1358.7) as shown in Table 3. The intraradical spore count was shown almost same as their percentage of root colonization (Figure 7) in MW medium (99204±1438.1, 75.4±2.50), M medium (89514±2272.1, 75.5±2.37) and MSR medium (82142±1059.3, 74.7±2.21) (Figure 6).

DISCUSSION

For large scale production of AMF inoculum in *in vitro*, so

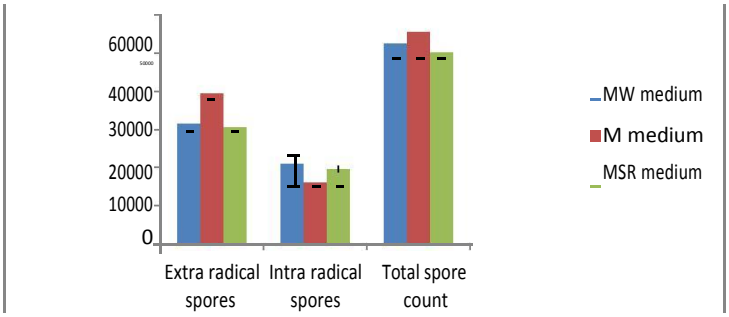

Figure 4. Spore count in containers with three different media.

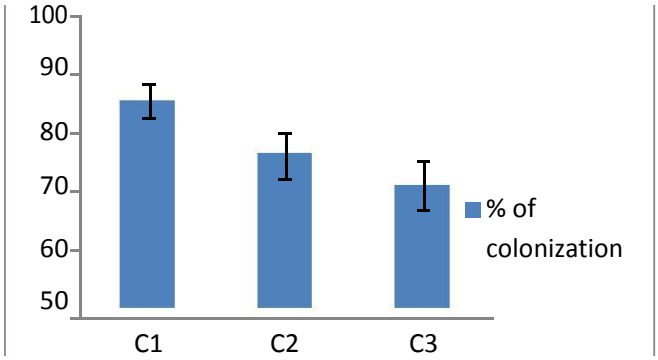

Figure 5. Percent of colonization in three different sucrose concentrations in M medium.

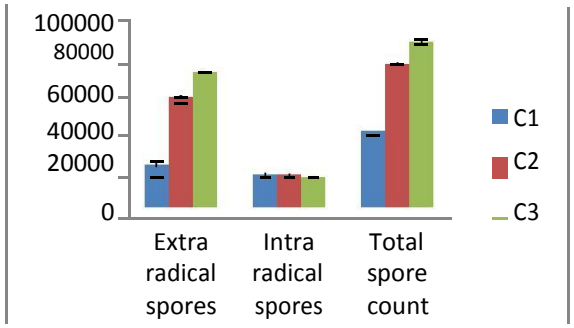

Figure 6. Spore count in three different sucrose concentrations in M medium.

much of research has been conducted from last two decades. The present study is also to develop a protocol for continuous large scale production of AMF spores, by modifying the root organ culture method with split plate method. To get the successful transformation, selection and identification of virulent *A. rhizogenes* bacterial strain is important. MTCC-532 (Chandigarh, India) strain successfully transformed the carrot roots. Continuous maintenance of this bacterial strain in YMA (Yeast Mannitol agar) medium in every 30 days intervals is also important.

Adholeya et al. (2005) developed *in vitro* protocol for large scale production of AMF through carrot root organ culture. Transformation with Tomato, Amaranthus, Carrot, Potato, Sweet potato and Soybean parts, carrot roots were gave very good success rate of transformation with *A. rhizogenes*. The source of carrots was also place an important role to get maximum success rate in transformation. Freshly harvested and actively growing carrots were shown almost 70% of success rate than the carrots from local markets. By placing the basal sides in upright position carrot slices shows very good response due to their endogenous auxin levels (Fusconi, 2014). Transformed carrot slices were formed the fresh white callus and hairy roots at cambial regions. To get bacterial free transformed roots, carbenicillin (500 mg) was shown immediate response within 3 to 4 subcultures (Nauutila et al., 1995).

The host reproducibility plays an important role in the continuous large scale production of AMF culture. For the continuous production of AMF inoculum callus root clumps (active host) were developed and continuously maintained in MS medium without losing their reproducibility. *R. irregularis* culture from R and D Centre for Conservation Biology and Plant Biotechnology was proliferated and inoculated easily to callus root clumps. For the continuous maintenance of active callus root clumps 3% of sucrose in MS medium was needed. With continuous subculturings in M medium, MSR medium and MW medium with 1 % sugars got necrosis and lost the reproducibility of hairy roots.

The mass production protocol achieved by Puri and Adholeya (2013) in jars made possible to reduce the cost and available to the farmers. St-Arnaud et al. (1996) was studied the variation in spore development in bi-compartment plate system. With the inspiration of split plate method container was separated by 0.4% CleriGel and 1% agar, it was made possible after solidification of phytagel covered with agar used MW medium (Figure 2E). St-Arnaud et al. (1996) got the tenfold higher spore in the sucrose free medium. In the present study, with the implementation of these two methods, 99204 total spore count was achieved within 90 days of incubation in MW medium.

Conclusion

In commercial scale production of AMF spores, continuous inoculums production is very crucial to run the cycle without disturbance and uniform production output. Regenerating callus root clumps will provide the active host continuously. Small variations in the spore count per container also make much difference in production cost, directly related to utilization of space and time. Selection of suitable medium among the three different media and

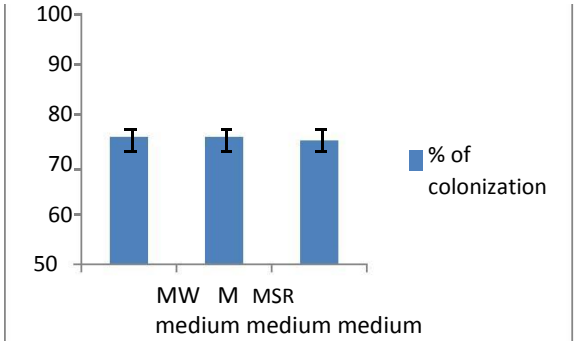

Figure 7. Percent of colonization in modified split plate method in 3 different media.

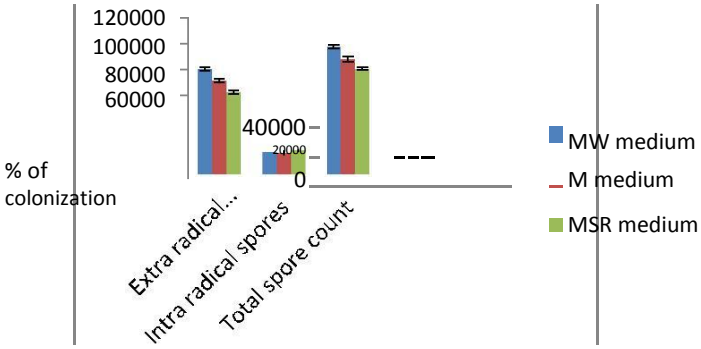

Figure 8. Spore count in modified split plate method in 3 different media.

implementation of split plate method, for continuous culturing of AMF spores make the choice of preference for large scale commercial production of AMF inoculums.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations: AMF, Arbuscular mycorrhizal fungus; **AM,** Arbuscular mycorrhizal; **M media,** minimal mineral media; **MSR media,** modified Strullu-Romand media; **Ri,** root inducing; **T- DNA,** transfer DNA; **MTCC,** microbial type culture collection; **LB,** Lauria-Bertani; **YMB,** yeast mannitol broth; **YMA,** yeast mannitol agar; **YEP,** yeast extract peptone; **MS,** Murashige and Skoog; **MW medium,** modified White's medium; **BOD,** biological oxygen demand.

REFERENCES

Adholeya A, Tiwari P, Singh R (2005). Large scale inoculum production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. In: Soil Biology, Volume 4 *in vitro* culture of Mycorrhizae

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(eds. Declerck S, Strullu –GD, Fortin A). Springer-Verlag Berlin Heidelberg 315-338.

Aggarwal A, Kadian N, Tanwar A, Yadav A, Gupta KK (2011). Role of arbuscular mycorrhizal fungi (AMF) in global sustainable development. J. Appl. Natural Sci. 3(2):340-351.

Aktar MW, Sengupta D, Chowdhury A (2009). Impact of pesticides use in agriculture: their benefits and hazards. Interdiscipl.Toxicol. 2(1):1-12.

Almeida-Rodríguez AM, Gómes MP, Loubert-Hudon A, Joly S, Labrecque M (2015). Symbiotic association between *Salix purpurea* L. And *Rhizophagus irregularis*: modulation of plant responses under copper stress. Tree Physiol. 36:407-420.

Ambrosini VG, Voges JG, Canton L, CoutoRda R, Ferreira PA, Comin JJ, de Melo GW, Brunetto G, Soares CR (2015). Effect of arbuscular mycorrhizal fungi on young vines in copper-contaminated soil. Braz. J. Microbiol. 46(4):1045-1052.

Anda CCO, de Boulois HD, Declerck S (2015). The arbuscular mycorrhiza fungus *Rhizophagus Irregularis* MUCL 41833 decreases disease severity of Black Sigatoka on banana c.v. Grande name, under in vitro culture conditions. Fruits 70(1):37-46.

Bago B, Azco´n-Aguilar C, Piche´ Y (1998a). Architecture and developmental dynamics of the external mycelium of th earbuscular mycorrhizal fungus I grown under monoxenic conditions. Mycologia 90:52-62.

Bago B, Vierheilig H, Piche Y, Azcon-Aguilar C (1996). Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol. 133:273-280.

Bécard G, Fortin A (1988). Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108:211-218.

Berruti A, Borriello R, Orgiazzi A, Barbera AC, Lumini E, Bianciotto V (2014). ArbuscularMycorrhizal Fungi and their Value for Ecosystem Management. Environmental Sciences » "Biodiversity - The Dynamic Balance of the Planet", (ed. Grillo O), Chapter 8.

Berruti A, Lumini E, Balestrini R, Bianciotto V (2016). Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. Rev. Front Microbiol. 6:1559.

Binondo LF, Pergola M, Silvani V, Colombo R, Bompadre J, Godeas A (2012). Continuous and long-term monoxenic culture of the arbuscular mycorrhizal fungus *Gigaspora decipiens* in root organ culture. Fungal Biol. 116:729-735.

Bompadre MJ, Pérgola M, Bidondo LF, Colombo RP, Silvani VA, Pardo AG, Ocampo JA, Godeas AM (2014). Evaluation of Arbuscular Mycorrhizal Fungi Capacity to Alleviate Abiotic Stress of Olive (*Olea europaea* L.) Plants at Different Transplant Conditions. Sci. World J. 2014(12).

Buysens C, César V, Ferrais F, Declerck S (2016). Inoculation of *Medicago sativa* cover crop with *Rhizophagu sirregularis* and *Trichoderma harzianum* increases the yield of subsequently-grown potato under low nutrient conditions. Appl. Soil Ecol. 105:137-143.

Cattani I, Beone GM, Gonnelli C (2015). Influence of *Rhizophagus irregularis* inoculation and phosphorus application on growth and arsenic accumulation in maize (*Zeamays* L.) cultivated on an arsenic-contaminated soil. Environ. Sci. Pollut. Res. Int. 22(9):6570-6577.

Ceballos I, Ruiz M, Fernández C, Peña R, Rodríguez A, Sanders IR (2013). The *In Vitro* Mass-Produced Model Mycorrhizal Fungus, *Rhizophagus irregularis*, Significantly Increases Yields of the Globally Important Food Security Crop Cassava. PLoS ONE 8(8):e70633.

De Andrade SA, Domingues AP , Mazzafera P (2015). Photosynthesis is induced in rice plants that associate with arbuscular mycorrhizal fungi and are grown under arsenate and arsenite stress. Chemosphere 134:141-149.

De Souza FA, Declerck S, Smith E, Kowallchuk GA (2005). Morphological, ontogenetic and molecular characterization of *Scutellospora reticulata* (Glomeromycota). Mycol. Res. 109:697-706.

Debiane D, Garcon G, Verdin A, Fontaine J, Durand R, Shirali P (2009). Mycorrhization alleviates benzo[a]pyrene-inducedoxidative stress in an *in vitro* chicory root model. Phytochemistry 70:1421-1427.

Declerck SD, Bivort C, De Souza FA (2004). Development of

extraradical mycelium of *Scutellospora reticulata* under root-organ culture: spore production and function of auxiliary cells. Mycol. Res. 108:84-92.
Declerck SD, Cranenbrouk S, Leboulenge E (2001). Modeling the sporulation dynamics of arbuscular mycorrhizal fungus in monoxenic culture. Mycorrhiza 11:225-230.

Dhawi F, Datta R, Ramakrishna W (2016). Mycorrhiza and heavy metal resistant bacteria enhance growth, nutrient uptake and alter metabolic profile of sorghum grown in marginal soil. Chemosphere 157:33-41.
Diop TA, Plenchette C, Strullu DG (1994a). Dual axenic culture of sheared-rood inocula of vesicular arbuscular mycorrhizal fungi associatedwith tomato roots. Mycorrhiza 5:17-22.
Driai S, Verdin A, Ed F, Sahraoui H (2015). Is the arbuscular mycorrhizal fungus *Rhizophagus irregularis* ableto fulfil its life cycle in the presence of diesel pollution? Int. Biodeterior. Biodegr. 105:58-65.
Fester T (2013). Arbuscular mycorrhizal fungi in a wetland constructed for benzene-, methyl tert-butyl ether- and ammonia-contaminated ground water bioremediation. Microb. Biotechnol. 6(1):80-84.
Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y (2002). Arbuscular mycorrhiza on root-organ cultures. Can. J Bot. 80:1-20.
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(1):19-33.
Gavito ME, Abud YC, Sántiz YM, Trujillo MM, Delgado CG, Rivera JRE (2014). Effects of aluminumand lead on the development of *Rhizophagus irregularis* and roots in root cultures. Environ. Eng. and Manag. J. 13(9):2357-2361.
Habibzadeh Y (2015). The effect of arbuscular mycorrhizal fungi and phosphorus levels on dry matter production and rrot traits in cucumber (*Cucumis sativus* L.). Afr. J. Environ. Sci. Technol. 9:65-70.

Hijri M (2016). Analysis of a large dataset of mycorrhiza inoculation field trials on potato shows highly significant increases in yield. Mycorrhiza pp. 209-214.
Ijdo M, Cranenbouck S, Declerck S (2011). Methods for large-scale production of AM fungi: past, present and future. Mycorrhiza 21:1-16.
Ingrid L, Lounès-Hadj Sahraoui A, Frédéric L, Yolande D, Joël F (2016). Arbuscular mycorrhizal wheat inoculation promotes alkane and polycyclic aromatic hydrocarbon biodegradation: Microcosm experiment on aged-contaminated soil. Environ. Pollut. 213:549-560.
Labidi S, Calonne M, Jeddi FB, Debiane D, Rezgui S, Laruelle F, Tisserant B, Grandmougin-Ferjani A, Sahraoui ALH (2011). Calcareous impact on arbuscular mycorrhizal fungus development and on lipidperoxidation in monoxenicroots. Phytochemistry 72:2335-41.
Lenoir I, Fontaine J, Sahraoui AL-H (2016). Arbuscular mycorrhizal fungal responses to abiotic stresses: A review. Phytochemistry 123.
Liu T, Sheng M, Wang CY, Chen H, Li Z, Tang M (2015). Impact of arbuscular mycorrhizal fungi on the growth, water status, and photosynthesis of hybrid poplar under drought stress and recovery. Photosynthetica 53(2):250-258.
Lone MI, He Z-I, Stoffella PJ, Yang X-e (2008). Phytoremediation of heavy metal polluted soils and water: Progresses and perspectives. J. Zhejiang Univ. Sci. B 9(3):210-220.
McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115(1):495-501.
Moreira H, Pereira SI, Marques AP, Rangel AO, Castro PM (2015). Mine land valorization through energy maize production enhanced by the application of plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi. Environ. Sci. Pollut. Res. Int. 6940-50.
Mosse B, Hepper CM (1975). Vesicular-arbuscularinfections in root organ cultures. Physiol. Plant Pathol. 5:215-223.
Mugnier J, Mosse B (1987). Vesicular-arbuscularinfections in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045-1050.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures Physiol. Plant 15:473-497.
Nuutila AM, Vestberg M, Kauppinen V (1995). Infection of hairy roots of strawberry (*Fragaria* × *Ananassa* Duch.) with arbuscular mycorrhizal fungus. Plant Cell Rep. 14(8):505-9.
Oruru MB and Njeru EM (2016). Upscaling arbuscular mycorrhizal symbiosis and related agroecosystems services in smallholder farming systems. BioMed. Res. Int. 2016:1-12.
Puri A, Adholeya A (2013). A new system using *Solanum tuberosum* for the co-cultivation of *Glomus intraradices* and its potential for mass producingspores of arbuscularmycorrhizal fungi. Symbiosis 59:87-97.
Schultze M (2013). Protocols for growing plant symbioses; mycorrhiza. Methods Mol. Biol. 953:47-59.
St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an *in vitro* system in the absence of host roots. Mycol. Res. 100:328-332.
Tahat MM, Kamaruzaman , Sijam, Othman R (2010). mycorrhizal fungi as a biocontrol agent. Plant Pathol. J. 9:198-207.
Takeda N, Handa Y, Tsuzuki S, Kojima M, Sakakibara H, Kawaguchi M (2015). Gibberellin regulates infection and colonization of host roots by arbuscular mycorrhizal fungi. Plant Signal Behav. 10(6):e1028706.
Tekaya M, Mechri B, Mbarki N, Cheheb H, Hammami H, Attia F (2016). Arbuscular mycorrhizal fungus *Rhizophagus irregularis* influences key physiological parameters of olive trees (*Olea europaea* L.) and mineral nutrient profile. Photosynthetica pp. 1-10.
Tiwari P, Adholeya A (2002). *In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. FEMS Microbiol. Lett. 206:39-43.
Voets L, De La Providencia IA, Fernandez K, Ijdo M, Cranenbrouck S, Declerck S (2009). Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. Mycorrhiza 19:347-356.
Wahbi S, Prin Y, Maghraoui T, Sanguin H, Thioulouse J, Oufdou K, Hafidi M, Duponnois R (2015). Field application of the mycorrhizal fungus *Rhizophagus irregularis* increasesthe yield of wheat crop and affects soil microbial functionalities. AJPS 6(19):3205-3215.
White PR (1943). A handbook of plant tissue culture. J. Cattel, Lancaster, Pa.
Wu S, Zhang X, Sun Y, Wu Z, Li T, Hu Y, Lv J, Li G, Zhang Z, Zhang J, Zheng L, Zhen X, Chen (2016). Chromium immobilization by extra- and intra-radical fungalstructures of arbuscular mycorrhizal symbioses. J. Hazard. Mater. 316:34-42.