Mass Propagation of *Echinacea angustifolia*: A Protocol Refinement using Shoot Encapsulation and Temporary Immersion Liquid System

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Abstract

Tissue culture of *Echinacea angustifolia* allows for selection and rapid multiplication of genetically superior clones. In this study, temporary immersion liquid bioreactor (RITA® system), synthetic seed technology and semi-solid culture conditions were evaluated for shoot multiplication efficiency. After 25 days of culture, the highest multiplication rate was observed in explants cultured in temporary immersion liquid system. Previous reports on micropropagation of *E. angustifolia* have had low success rate in rooting. Shoots were rooted under autotrophic conditions on sugar-free semi-solid media supplemented with 2.5 μM IBA and using synthetic seed technology by shoot encapsulation in calcium alginate gel cultured on MS medium supplemented with 2.6 μM NAA. Rooting response was noticed in three weeks with a success rate of 83%. Arbuscular mycorrhiza infection helped the acclimatization survival rate of rooted plantlets.

INTRODUCTION

Echinacea is one of the most popular herbal supplements among the general public in the United States and the popularity of this medicinal plant is due to its use in the treatment of infections and colds, based on its immune-stimulant and anti-inflammatory effects (Giles et al., 2000). The current sales of *Echinacea* are in $39 million per year according to Blumenthal (2002) market report.

Several clinical studies on the therapeutic use of *Echinacea* have demonstrated the benefits on recovery and prevention of infections (Giles et al., 2000 and Lindenmuth and Lindenmuth, 2000), but other studies have failed to show efficacy (Mark et al., 2001 and Vonau et al., 2001). Lack of product standardization and consistency within protocols are possible reasons for these inconsistencies in clinical studies (Melchart and Linde, 1999). For commercial preparations of *Echinacea*, roots and foliage of three different species (*Echinacea purpurea*, *Echinacea angustifolia* and *Echinacea pallida)* have been wild crafted or cultivated. Thus, authenticity and genetic identity are important issues that may affect quality, safety and efficacy of the final products. Variation among the final product formulations (encapsulated powder, expressed juices and tinctures) may lead to different chemical compositions, which may also contribute to these inconsistencies.

As the popularity of *Echinacea* as a dietary supplement depends on quality of the products, selection of superior genotypes based on biological activity is of interest. Developing a mass propagation protocol to rapidly increase superiors *E. angustifolia* genotypes is the objective of this study. Choffe et al (2000) have reported a successful micropropagation protocol of *E. purpurea* but rooting has been a challenge for mass propagation of *E. angustifolia* (Harbage, 2001). Many plant species produced in nutrient-rich substrate under sterile conditions fail to survive the transference to soil conditions. Inoculation by arbuscular mycorrhizal fungi (AMF) has aided the survival and growth of these species during plantlet acclimatization (Azcon-Aguilar et al.1996, Salamanca et al. 1992). Further improvements in the existing *E. angustifolia* micropropagation protocol are needed including optimization of the in vitro microenvironment and the survival rate during acclimatization. In this study, we are attempting to improve the culture conditions

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by using temporary immersion liquid system and acclimatization of plantlets by mycorrhization, refining *E. angustifolia* protocol of mass propagation.

MATERIALS AND METHODS

Plant Material

All experiments were conducted with shoot cultures obtained from germinated seeds using hypocotyl as an explant. The North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa provided seeds of *E. angustifolia* (PI 312814 accession). Prior to inoculation on the germination media, seeds were surface disinfected by washing with 1% NaOCl (20% v/v bleach) and 0.1% Tween 20 for 10 minutes, followed by washing three times in sterile distilled water (5 minutes per wash). Aseptic seedlings were initiated on agar (0.8%) solidified half strength MS medium (Murashige and Skoog, 1962) without sucrose using ventilated closures. Cultures were incubated at 25 ± 2° C, 16h photoperiod under fluorescent light with a photon flux of approximately 52μmol. $m^{-2}.s^{-1}$.

After 10 days, 0.5-1.0 cm long hypocotyl were taken as explants for the initiation of shoot cultures. Shoot cultures were obtained on MS half strength (MS/2) macronutrients medium containing 3% (w/v) sucrose, 0.8% (w/v) agar supplemented with 2.2 μM of 6 Benzyladenine (BA) per liter, adjusted to pH 5.7. The same multiplication medium was used in the three cultured systems described below.

Shoot Multiplication

Echinacea shoots were transferred into three different culturing systems for shoot multiplication: temporary immersion system (RITA® bioreactor), encapsulated shoots using synthetic seed technology and semi-solid culture conditions. For the temporary immersion, 15 explants were inoculated on the upper compartment of each RITA® system in each of three bioreactors. Each replicate consisted of three explants removed at 5-day interval per RITA® system during the growing period of 25 days. For synthetic seed technology, each explant was encapsulated in 5% sodium alginate and 50 mM $CaCl_2$ producing individual beads. Three individually encapsulated shoots were cultured per vessel on semi-solid MS media supplemented with 2.2 μM BA. A total of 15 vessels composed the encapsulated shoot treatment with three replicates. Same arrangement was used for the cultures on semi-solid media supplemented with 0.8% (w/v) agar.

Rooting

Shoots were transferred onto rooting media maintaining under the same conditions as described. Treatments were replicated six times in rooting, each RITA®system contained 15 shoots per bioreactor. For the synthetic seed technology, three encapsulated shoots were inoculated per vessel. Three shoots per vessel were also transferred to semi-solid culture condition. An additional treatment was tested for rooting under autotrophic condition using semi-solid sugar free media with vented closures.

In rooting *E. angustifolia* under these different culture conditions, both MS and MS/2 macronutrients supplemented with three different auxins; 2.6 μM NAA (α-naphthalene acetic acid), 2.5 μM IBA (indole-3-butyric acid) and 2.8 μM IAA (indole-3-acetic acid) were tested.

Rooted and un-rooted plantlets were rinsed with sterile water to remove residual rooting medium and transferred to planting tray kits with 6 cells and a plastic dome and based tray marketed by Wal-Mart Stores, Inc. for acclimatization to soil.

Fungal Inoculums

Inoculums of *Glomus mosseae* (Nicol and Gerd) Gerd and Trappe, *Gigaspora ramisporophora* Spain, Sieverding and Schenck, *Scutellospora fulgida* Koske and Walker, Entrophospora *colombiana* Spain and Schenk were provided by Dr. Gisela Cuenca of Instituto Venezolano de Investigaciones Científicas, Caracas Venezuela.

Acclimatization

For establishment of mycorrhizal association with the hardened plantlets of *E. angustifolia,* the inoculums of the four species were mixed and each micropropagated plantlet received 10 g of inoculums corresponding to 450-600 spores, placed adjacent to and below the roots into the soil at planting time. Soil substrate used was a mixture of (1:2, v/v) potting soil (Potting Mix Miracle Gro 0.14 0.14 0.14) and sand (Garden Basic Play Sand, Sims Bark Co, Tuscumbia, AL)

The plantlets were maintained in a glasshouse at the Biological Field Station, University of Mississippi under a mist system with 10 minutes watering cycle every 20 minutes for a period of 10 hours. The performance of the plantlets was evaluated after 30 days by measuring transplanting percentage survival, shoot and root length.

RESULTS AND DISCUSSION

In vitro plant propagation on semisolid media involves a considerable amount of contamination risks and tissue damage due to handling (Weathers and Giles, 1988). The highest shoot multiplication rate was observed using temporary immersion of explants in the RITA® system. Shoot induction in RITA® system was two-fold increase in multiplication rate compared to encapsulated shoots and the semi-solid media, demonstrating the advantages of the temporary immersion of explants in a liquid medium. While inclusion of liquid cultures for scaling up the production may be cost effective, the vitrification generally limits the process (Debergh et al., 1981). Growing E. *angustifolia* using the temporary immersion did not show vitrification. Our findings corroborate the positive reports on the use of this technology for other species such as banana (Alvard et al., 1993), microcuttings of *Elaeis guineensis*, *Eucalyptus globules* (Teisson et al., 1996), *Citrus deliciosa* (Cabasson et al., 1997) and *Coffee arabica* (Etienne-Barry et al., 1999).

For rooting *E. angustifolia,* a preliminary study was done by transferring shoots onto MS media with different concentrations of macronutrients with varying levels of auxin (NAA, IBA and IAA). Additional supplements of phloroglucinol and dithiothreitol (DTT) were also tested. Rooting was unsuccessful for most of the treatments with the exception of MS/2 semi-solid media supplemented with 2.6 μM NAA (data not shown). Thus, a second trial with fewer media ingredients under various culture conditions was evaluated. Only positive results in *E. angustifolia* rooting are presented (Table 1). Rooting results demonstrated that supplement of sucrose in semi-solid media was not an essential component for rooting (Table 1). Root induction was lower in sugar containing media than in autotrophic conditions. Lack of sugar in the semi-solid condition may have prevented production of substances that could be harmful to rooting.

The use of encapsulated shoots is a promising procedure regarding vigor and number of roots per plantlet, although, the percentage of rooted plantlets was higher in autotrophic cultures (Table 1). The use of shoot encapsulation, in this study was modest, just an attempt to improve rooting of *E. angustifolia* micropropagated plantlets. Several aspects of synthetic seed technology is still a potential strategy to be pursed in *Echinacea* such as encapsulation of somatic embryos or encapsulation of microclones for soil adaptation. The RITA® system using temporary immersion in liquid media was not an efficient rooting system for *E. angustifolia.* Callus growth was induced at the base each shoot for most of the auxin treatments and it did not differentiate in roots.

Rooting *E. angustifolia* on semi-solid sugar free media was an effective procedure and a necessary step for adaptation to soil condition. Only 40% of the un-rooted plantlets survived transplantation after 30 days. In un-rooted plantlets, the rate of survival was improved by early mycorrhizal inoculation reaching the success rate of 60%. Early mycorrization was even more effective on rooted plantlets with the survival of 83% within 30 days (Fig. 2). In addition, we found that mycorrizal rooted plantlets were more vigorous with taller plants (3.0 cm) and longer roots (1.5 cm) in average (Fig. 3). According to Pons et al., (1983) axenic culturing conditions take away normal symbiotic interactions between plant and soil microflora and in vitro propagated plantlets have limited resistance to stress during the transplant process, which can be restored by early

mycorrhizal inoculation as it was done in this study.

In conclusion, the combination of different culturing systems as such as RITA® system for shoot multiplication, semi-solid sugar free media with vented closures for rooting and inoculation of arbuscular mycorrhiza at acclimatization produced vigorous *E. angustifolia* soil adapted plants.

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Tables

Table 1. Evaluation of different types auxins, concentrations of macronutrients in MS salts and culture systems on rooting of *Echinacea angustifolia.*

Auxin*	Culture system	MS salts Conc.	No. of roots per shoot	Root length cm	% of rooted plantlets
NAA	Semi-solid	MS	5.5 ± 1.2	0.3 ± 0.1	33
NAA	Semi-solid	MS/2	4.0 ± 0.8	0.9 ± 0.4	66
NAA	Autotrophic	MS/2	3.0 ± 0.9	1.6 ± 0.5	17
IAA	Autotrophic	MS/2	3.6 ± 1.3	2.1 ± 0.8	50
IBA	Autotrophic	MS/2	5.0 ± 0.7	1.5 ± 0.6	83
NAA	Encapsulated	MS	6.0 ± 0.8	4.3 ± 0.9	75
IAA	Encapsulated	MS	5.5 ± 1.0	3.6 ± 0.7	50
IBA	Encapsulated	MS	7.0 ± 1.5	3.0 ± 0.5	17

*Mean represents six replicates for each treatment, followed by standard error.

Figures

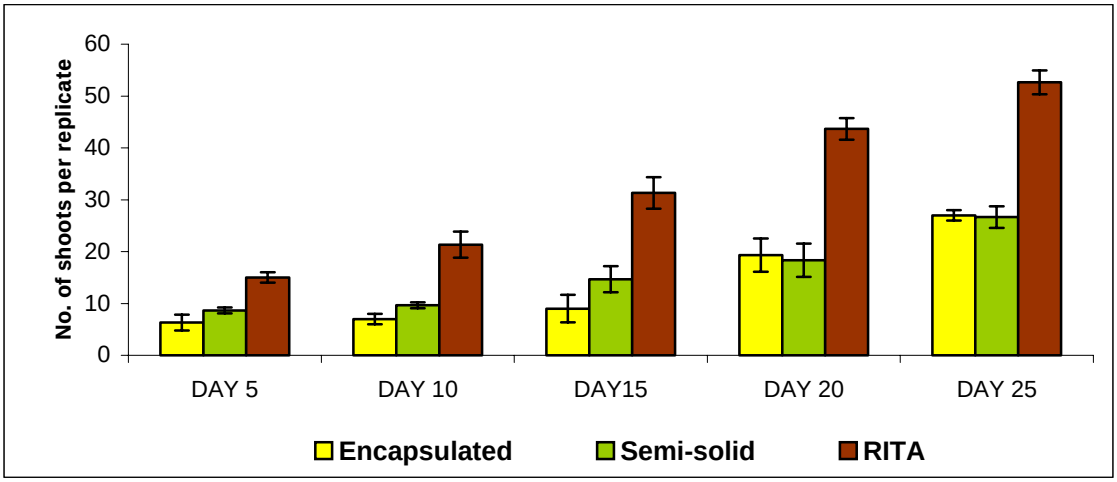

Fig. 1. Comparison of *Echinacea angustifolia* explants cultured in three different conditions encapsulated shoots using synthetic seed technology, semi-solid media and temporary liquid immersion system (RITA®). Data represent the mean of three replicates with three explants per treatment.

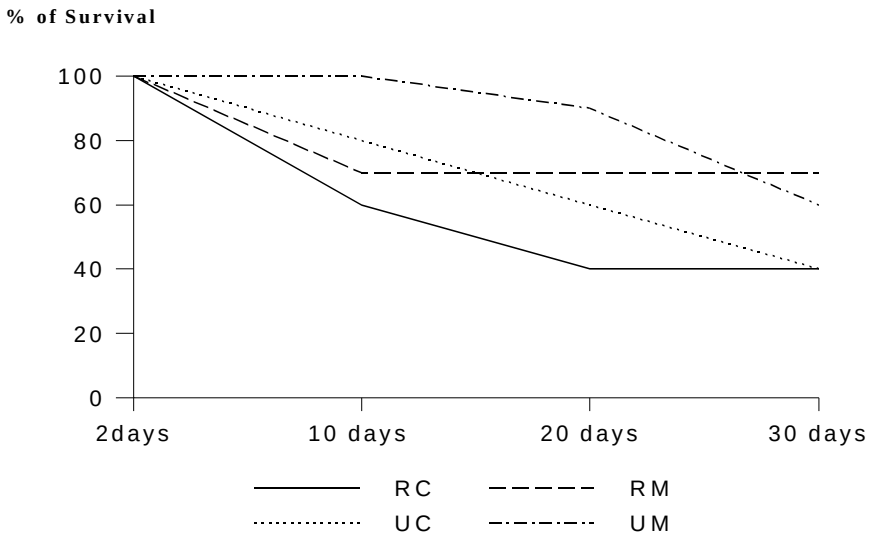

Fig. 2. Acclimatization and survival rate of rooted non-mycorrhizal (RC), rooted mycorrhizal (RM), un-rooted non-mycorrhizal (UC), un-rooted mycorrhizal (UM) micropropagated plantlets of *Echinacea angustifolia.*

Fig. 3. Comparison of unrooted non-mycorrhizal, unrooted mycorrhizal, rooted non-mycorrhizal, rooted mycorrhizal (left to right) micropropagated plantlets of *Echinacea angustifolia.*