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Full Length Research Paper

Application of artificial seeds in rapid multiplication of *Pseudostellaria heterophylla*

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In this study, a method which produces artificial seeds of *Pseudostellaria heterophylla* was presented. The micro-tubers induced from virus-free seedlings were used as explants, and the coating material was sodium alginate. The results show that the appropriate medium for *P. heterophylla* micro-tubers formation was the MS medium supplemented with 2.0 mg l^{-1} NAA, 500 mg l^{-1} CCC and 50 g l^{-1} sucrose; and the average number of micro-tuber reached 7.3 in each seedling under these conditions. Moreover, the optimum medium for the highest germination rate (83.8%) was 1/4 MS (without sugar) + 1.0 mg L^{-1} 6-BA + 0.1 mg L^{-1} NAA, and the artificial seeds were more perishable in sucrose-rich environments. It also indicated that the artificial seeds were still active with a seedling rate of 80% after they were preserved at 4°C for 90 days, which could meet the requirement of winter-produced seeds and plants in spring.

Key words: Artificial seed, artificial endosperm, micro-tuber, *Pseudostellaria heterophylla*, sodium alginate.

INTRODUCTION

Pseudostellaria heterophylla is a valuable Chinese traditional herb used for hundreds of years in China to help people recover from chronic illnesses. With a rise in market demand, the wild resources are going into extinction due to over exploitation. Generally, the *P. heterophylla* roots sold in the markets are usually collected from farms by root tubers asexual propagation; however, the yield and quality are often visibly reduced due to virus diseases (Wu et al., 2006).

At present, the seed root of *P. heterophylla* is mainly produced in the field. Seed root cultivation in soil is time-consuming, easily interfered with climate and subject to re-infection by virus (Lin et al., 2004). Artificial seed production is a potential technique for plant multiplication and preservation, especially as it has been considered to be promising for propagation of no-seed producing plants, transgenic plants and other plants that need to keep superior traits by means of asexual propagation (Saiprasad, 2001). Plant artificial seed in a narrow sense, means the beads formed by encapsulating somatic embryo with coating materials. Its effect varied with different species, coating materials, maintained solutions and its concentration and condition (Nhut et al., 2005). Murashige (1978) firstly proposed the use of somatic embryo for plant propagation and put forward the concept of artificial seed. However, artificial seed, in a narrow sense, is only a feasible mean for certain plants that can be of low production (Slade et al., 1989). Kamada (1985) presented a general concept of plant artificial seed, in which all kinds of plant explants with germination ability can be used for artificial seed production. Nowadays, it is widely used in many plants (Slade et al., 1989; Fukai et al., 1994; Stephen and Jayabalan, 2000; Ipekci and Gozukirmizi, 2003; Halmagyi et al., 2004; Nhut et al., 2005). Different coating materials were tested such as hydrogel sodium alginate, polyethyleneimine and chitosan (Fujii et al., 1987; Redenbaugh et al., 1991; Kersulec et al., 1993; Tay et al., 1993). Furthermore, different preservation

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Abbreviations: MS, Murashige and Skoog medium **NAA,** 1-naphthalene acetic.

Table 1. Effect of different phytohormones concentration on the formation of micro-tuber.

S/N	A (NAA) (mg l^{-1})	B (CCC) (mg l^{-1})	C (sucrose) (g l^{-1})	D (Blank control)	Mean number of micro-tuber from each seedling
1	1 (2.0)	1 (700)	1 (80)	1	7.3
2	1	2 (500)	2 (50)	2	7.3
3	1	3 (100)	3 (20)	3	5.5
4	2 (1.0)	1	2	3	5.7
5	2	2	3	1	3
6	2	3	1	2	6.3
7	3 (0)	1	3	2	2.2
8	3	2	1	3	3.3
9	3	3	2	1	3.3

The experiments were designed by using an orthogonal test of L9 (3^4), and all results were repeated 3 times.

conditions were investigated (Datta et al., 1999; Nhut et al., 2005; Aitken-Christie et al., 1995).

Artificial seed production is an outstanding technique used to propagate and preserve plants and has been applied on many plants. However, only a few has been reported on researches on growth and the second production of *P. heterophylla* (Wang and Qi 2010). The present study focuses on the presentation of a method used to produce the artificial seed of *P. heterophylla*. The micro-tubers induced from virus-free seedlings were used as explants, and the coating material was sodium alginate. In addition, induction of microtubers, choice of artificial endosperm, as well as preservation conditions were also investigated.

MATERIALS AND METHODS

Fresh buds of *P. heterophylla* obtained from China Pharmaceutical University were surface disinfected for 8 min by 0.1% $HgCl_2$ solution and then immersed in 75% alcohol for 30 s. After it was rinsed three times in sterile distilled water, the growth points with one leaf primordium were placed on an MS (Murashige and Skoog, 1962) medium supplemented with 30 gl^{-1} sucrose, 5.5 g l^{-1} agar and 0.5g l^{-1} 6-BA to obtain virus-free aseptic seedlings. The pH value of the media was adjusted to 5.8 prior to autoclaving and the cultures were maintained at 25°C in the dark.

Induction of micro-tubers

To induce micro-tubers, the virus-free seedlings identified by electron microscope were transferred to a solid MS medium with different concentrations of 6-BA, CCC (Cycocel) and sucrose (Table 1). To optimize the growth regulators, an experiment was carried out with a $L_9(3^4)$ orthogonal array. The experiment incorporated 4 variables at 3 different settings with 3 replicates. The numbers of micro-tubers formed from each seedling were recorded 45 days after transfer to the inducing media. The culture conditions were kept at 25°C in the dark.

Artificial seeds production

The SA (sodium alginate) solution was prepared by slowly adding 25 g dry sodium alginate into 1 L 1/4 liquid MS medium, which was supplemented with 6-BA, NAA and sucrose with different concentrations, and then these ingredients were mixed until the solution became viscous. The $CaCl_2$ (Calcium Chloride) was prepared by adding 20 g calcium chloride into one liter distilled water, and then the pH was adjusted to 5.8. Micro-tubers, wrapped by sterilized semigel-SA solution, were sucked up into a modified 10 ml pipette, and then dropped into 2% $CaCl_2$ solution. Artificial seeds were immersed in $CaCl_2$ solution for 5 min in order for them to be hardened, after which they were picked out and washed with sterilized water. The process was based on the method of Nhut et al. (2005) with minor modification.

Preservation and seedling formation

After encapsulation, the artificial seeds were preserved in a closed sterile container at 4 and 25°C for 10, 30, 90 and 150 days, respectively. Seedling-forming test of the artificial seeds were also carried out with different levels of storage temperature and time intervals. Sterile culture was conducted with MS medium in a thermostatic chamber at 25°C. For soil cultivation, artificial seeds were placed in soil pits with a depth of 3 cm in greenhouse for one month at 25°C.

RESULTS AND DISCUSSION

Optimization of micro-tuber induction

Roots were initiated and elongated after transferring seedlings to the inducing medium. At the same time, adventitious roots were formed from the nodes. With normal roots and adventitious roots enlarging, micro-tubers were formed. The number and time of micro-tubers formation have different optima for plant growth regulators. The optimum factorial combination for *P. heterophylla* micro-tubers induction was $A^1B^2C^2$ (MS media supplemented with 2.0 mg l^{-1} NAA , 500 mg l^{-1} CCC and 50 g l^{-1} sucrose), and the average number of micro-tuber reached 7.3 in each seedling under this condition (Table 1).

Analysis of variance (Table 2) indicated that micro-tuber number increased in response to higher concentrations of

Table 2. Variance analysis of micro-tuber formation.

Source of variance	Square of deviance	Degree of freedom	F value	$F_{0.01}$
S_A	21.349	2	2.8	4.46
S_B	0.536	2	0.07	4.46
S_C	7.796	2	1.023	4.46
S_D	30.5	8		

S_A, S_B, S_C and S_D refer to the source of variances from the concentration of NAA, CCC, sucrose and blank control, respectively.

Table 3. Effect of additives on the germination of artificial seeds.

S/N	Nutrient and growth regulator	Average germination rate (%)
1	Distill water	79
2	1/4 MS_0	78.3
3	1/4 MS + sucrose 20 g l^{-1}	66.2
4	1/4 MS + 6-BA 1.0 mg l^{-1} + NAA 0.1 mg l^{-1}	83.8
5	1/4 MS + 6-BA 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} + sucrose 20 g l^{-1}	72.6

The artificial seeds stored at 4°C for three months were planted in the soil, and the germination rate was calculated after 30 days of incubation at 25˚C. 1/4 MS_0 means the ms media with 1/4 quantity of macronutrients and without additives.

NAA and sucrose, but both of them had no significant influence on micro-tuber formation.

Sucrose was prone to promoting the formation of adventitious and fibrous roots, but excessive rooting did not increase the number of micro-tubers. The *P. heterophylla* seedlings propagated so rapidly that they grew fully in the culture bottle within four weeks. Additional CCC to the medium could strengthen seedlings and lengthen subculture intervals, although, no increase was observed in the number of micro-tubers. Interestingly, micro-tubers were also formed after a long time (four months) of culture in the medium with no growth regulators.

Effect of artificial endosperm on germination of artificial seed

Micro-tubers lack seed coat and endosperm that provide protection and nutrition. Therefore, the nutrients and phytohormone were added to the encapsulation agent that served as an artificial endosperm. However, the results indicate that additives had no significant relationship with efficiency of germination, which may be attributed to the nutrition that was self-supplied by the root micro-tubers (Table 3). Artificial seeds with No. 4 additives of 1/4 MS with 1.0 mg l^{-1} 6-BA and 0.1 mg l^{-1} NAA were used as the coating material, while the germination rate was up to 83.8%; although, shoots were also formed without any additives. In addition, the germination rate was enhanced through the supplying of sucrose to the artificial endosperm. On the contrary, micro-tubers were more perishable in sucrose-rich environment. This research also showed that the artificial seeds can be preserved for a long period of time without losing viability at 4°C (Table 3).

Effect of storage temperature on the seedling formation

One of the advantages of artificial seed production is that it is not subject to seasonal restrictions. Generally, winter-produced seeds can be planted in spring; therefore, it is essential for preservation in a long period of time without loss of activity. In this study, different storage temperature and storage times were designed to determine the appropriate preservation conditions. The results show that storage time of artificial seeds at room temperature (25°C) cannot exceed 30 days; otherwise, the seedling rate will decrease visibly. After preservation at 4°C for 90 days, artificial seeds were still active with a seedling rate of 80%, which could meet the requirement of winter-produced seeds in spring planting use. The seedling rate of sterile culture was higher than that of soil cultivation, thus, it may also decline slightly in field planting as the temperature cannot be controlled (Figure 1).

It is difficult to meet the low-temperature requirement for vernalization of *P. heterophylla* in Southern China (Wen et al., 2003) which is essential for seed setting. Therefore, the roots of *P. heterophylla* were generally used as reproductive organs to maintain the superior traits. In contrast to other *in vitro* organs (Nhut et al., 2004; Chand and Singh, 2004; Danso and Ford-lloyd, 2003; Kamada et al., 1998), root micro-tubers as explants of artificial seed have the advantages of easy fabrication, such as: low cost, long time storage and high frequency

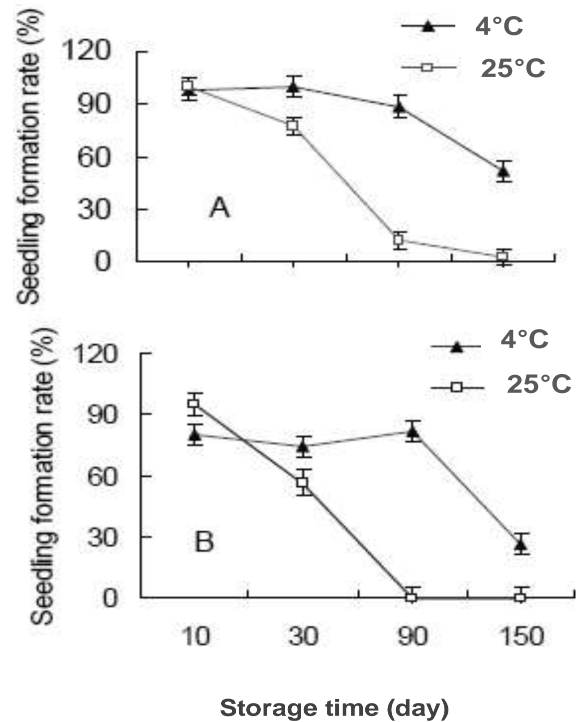

Figure 1. Effect of storage time on seedling formation of artificial seed. (A) Sterile culture. (B) Soil culture. The medium of 1/4 MS containing 1.0 mg l^{-1} 6-BA and 0.1 mg l^{-1} NAA was used as coating material. Ten artificial seeds with different storage temperature and storage time were planted in the soil and sterile culture bottle respectively, as well as the seedling formation rate was calculated after 30 days of incubation at 25˚C.

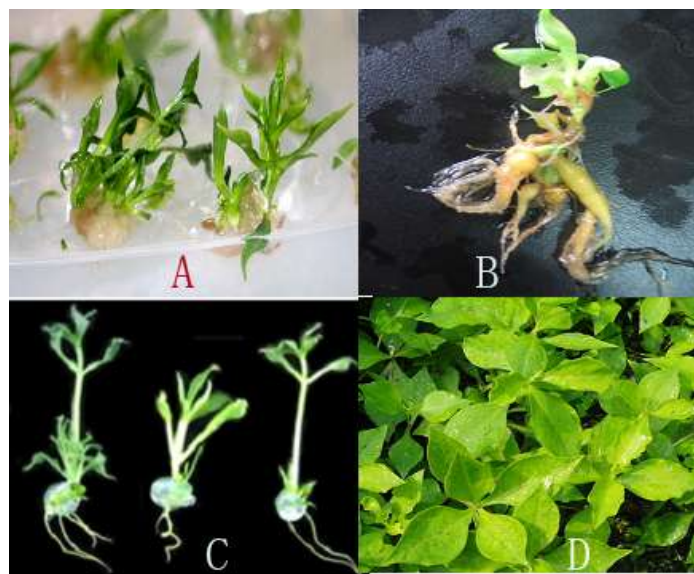

Figure 2. Development and culture of P. heterophylla adventitious roots. (A) Seedlings of *P. heterophylla*. (B) Micro-tubers developing from virus-free seedlings. (C) Plantlets regenerated from artificial seeds by sterile culture. (D) Plantlets regenerated from artificial seeds by soil culture.

of plantlets regeneration. The further spreading and application of artificial seed is based on many factors, including the efficiency of the existing explants regeneration system, relative cost of a specific application for a given plant species, etc. For example, the synthetic seed of seedless watermelon would be less costly than the conventional seed (Saiprasad, 2001). The results of this study showed that *P. heterophylla* artificial seed production and the formation of *in vitro* plants derived from these synthetic seeds were feasible, and the benefit that could be conferred by their use would be very great. Additional researches are needed to promote the artificial seeds of *P. heterophylla* in order for them to be commercialized (Figure 2).

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