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A Double Phase Culture System: An Economic and Time Saving Protocol for *In Vitro* Propagation of Plant

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Abstract

Low cost and time saving tissue culture techniques by improving process efficiency and better utilization of resources without compromising the quality of the plants are of high priority for commercial utilization. In this report a double phase culture system (DPS) i.e. a semisolid phase with rooting media at the bottom with a layer of shooting media in liquid phase, was standardized and compared with conventional micro propagation system (CMS) by taking *Rauwolfia serpentine* L. Bent. as a model. In CMS the shoots were raised first followed by two subsequent subculture then they were transferred to rooting media for root induction for which it will take more than 12 weeks but under DPS, plantlets with well developed roots and shoots can be produced simultaneously in a single culture vessel within 08 weeks without shoot manipulations/repeated subculture. It was found that the rate of multiplication (98.7), shoot length (9.8) and shoot thickness (5.2) was higher in DPS in comparison to CMS. The no. of roots/shoot (8.7), root length (5.3cm), root thickness (6.1mm) was found higher in DPS than that of the CMS. Percentage survival of plantlets was 98.5 % in DPS, while it was only 70% in CMS. The culture period was reduced by 44% in DPS compared to the CMS. In DPS nutrient cost/plantlet, energy cost/plantlet and labour cost also reduced by 35.36%, 40.66% and 33.33% respectively in comparison to CMS. Thus DPS has many advantages and can be used for large scale quality production of commercial plants in reduced time, cost and labour, which ultimately reduced the production cost of the farmer.

Keywords: DPS; CMS; *Rauwolfia serpentine*; Economical value

Introduction

Indiscriminate over exploitation from natural source to meet the growing demand by industry coupled with low seed viability, lack of vegetative propagation methods and insufficient attempts for replenishment of wild stock of different important plant species have contributed to its threatened status. So realizing the threat of extinction and to meet the growing needs, the alternate method for propagation is micropropagation. This technique is used not only for those plants which are difficult to be propagated through conventional practices, but also for the mass multiplication of existing stocks of germplasm for biomass production and conservation of important, elite and rare plant species that are threatened or on the verge of extinction [1]. In all reports, protocols described are based on conventional micropropagation system (CMS). This CMS explained most common methods of micropropagation (Table 1), which involve initiation/bud proliferation, the shoot multiplication followed by rooting via a semi solid medium. Although CMS have been highly successful in terms of multiplication yields, it has become increasingly important to improve productivity and uniformity of commercially important material in reduced cost, time and space [2]. Hence an improvement in plant tissue culture techniques for the mass propagation of commercial plants is highly desirable [3]. An alternative that has shown potential to improve the efficiency of protocols for *in vitro* multiplication is the use of a double-phasic culture system (DPS). In this method, the explants remain in the semi-solid medium and the liquid medium is added over periodically throughout the culture, thus

eliminating the need of subculture the labour cost and the chance of contamination during subculture can be avoided. The use of culture space and culture vessel can be reduced so that using minimum culture space and minimum no. of culture vessel large no. of plants can be propagated. To date a very few reports on DPS has been reported that has improved the efficiency of *in vitro* shoot development in certain plants like in Pineapple [4], in conifers [5] and in Japanese pear [6]. All these DPS study, reported same media composition (both in the semi-solid and liquid phase) responsible for increasing the efficiency of shooting only. However, hardly any report on DPS involves the use different media i.e., rooting and shooting in the same culture vessel. A double phase

culture system (DPS) (Semisolid phase with rooting media with a layer of shooting media in liquid phase) was used to study the effect of shooting and rooting simultaneously. The objective of the present study is to test the feasibility of this DPS protocol and determine its efficiency of plant production in comparison to CMS by taking *Rauwolfia serpentine* L. Benth. (family- Apocyanaceae) commonly known as sarpagandha as a model due to its high medicinal value and heavy demand in pharmaceutical industry. More than 30 indole alkaloids (0.7–2.4%), phytosterols, unsaturated alcohols and sugars have been isolated from this plant. Among various alkaloids, reserpine is the most active constituent and is well known for its antihypertensive nature [7]. Extracts from different parts of this plants is widely used in ayurvedic system of medicine against many diseases such as hypertension, high blood pressure, insomnia, schizophrenia, insanity, epilepsy, hypochondria, breast cancer, diarrhoea, and disorders of the central nervous system [8].

Stages of micropropagation	Culture media used	Tissue culture responses	
		CMS	DPS
Bud proliferation	MS +3% sucrose	00	00
	MS + 0.10mg/l BAP + 3% sucrose	67 ± 0.6b	68 ± 0.5b
	MS + 0.25mg/l BAP + 3% sucrose	76 ± 0.5c	79 ± 0.7c
	MS + 0.50mg/l BAP + 3% sucrose	96 ± 0.6e	97 ± 0.5e
	MS + 1.00mg/l BAP + 3% sucrose	56 ± 0.7a*	63 ± 0.4a*
	MS + 0.50mg/l Kn + 3% sucrose	86 ± 1.0d	89 ± 0.8d
	MS + 0.5mg/l BAP + 0.50mg/l Kn + 3% sucrose	90 ± 0.8e*	91 ± 0.7e*
Shoot multiplication and growth	MS + 0.5mg/l BAP + 3% sucrose	32.6 ± 0.8a	36.3 ± 0.8a
	MS + 1.5mg/l BAP + 3% sucrose	51.2 ± 0.7b	55.2 ± 0.5b
	MS + 2.0mg/l BAP + 3% sucrose	76.5 ± 0.8d	81.4 ± 0.6e
	MS + 2.5mg/l BAP + 3% sucrose	60.3 ± 1.0b*	63.3 ± 0.8c*
	MS + 2.0mg/l Kn + 3% sucrose	66.3 ± 0.5c	71.3 ± 0.5d
	MS + 2.0mg/l BAP + 0.1mg/l NAA + 3% sucrose	79.2 ± 0.8d	85.2 ± 0.8e
	MS + 2.0mg/l BAP + 0.25mg/l NAA+ 3% sucrose	96.4 ± 0.8e	98.7 ± 0.4f
	MS + 2.0mg/l Kn + 0.25mg/l NAA + 3% sucrose	70.2 ± 0.6d	76.2 ± 0.7d
Root induction and growth	½ MS +0.25mg/l IAA + 2% sucrose	44.2 ± 0.8 b	48.2 ± 0.8 b
	½ MS + 0.50mg/l IAA + 2% sucrose	61.4 ± 1.0d	69.4 ± 0.7d
	½ MS + 0.25mg/l IBA + 2% sucrose	53.4 ± 0.8c	57.6 ± 0.8c
	½ MS + 0.50mg/l IBA + 2% sucrose	73.3 ± 1.0e	79.1 ± 0.7e
	½ MS + 1.00mg/l IBA + 2% sucrose	48.3 ± 0.6b*	51.6 ± 0.7b*
	½ MS + 0.50mg/l NAA + 2% sucrose	36.5 ± 0.6a	39.8 ± 0.8a
	½ MS + 0.50mg/l IBA + 0.25mg/l NAA + 2% sucrose	60.0 ± 0.8c	70.6 ± 1.0d
	½ MS + 0.50mg/l IBA + 0.25mg/l IAA + 2% sucrose	59.2 ± 0.6c*	63.5 ± 0.8d*

Values are mean ± SE; 20 replicates per treatment, repeated three times; *callusing at the basal end; means having a same letter within columns were not significantly different at $P < 0.05$.

Table 1: List of selected media used in the different stages of micropropagation in *Rauwolfia serpentine* and their responses

Materials and Methods

To test the feasibility of this DPS for culture *Rauwolfia serpentine*, nodal explants from the mature plants were collected from herbal garden of Department of Biotechnology, Guru Ghasidas Vishwavidyalaya, Bilaspur, India [9]. Elongated shoots (4–5 cm long) were collected, sterilized and inoculated in MS medium [10] supplemented with and without growth regulators by following the modified methods of [11]. After bud proliferation the explants (1.5-2.0 cm) were transferred to the DPS and CMS. In DPS two different phase i.e. semisolid phase and liquid phase with two different media composition were used. The semisolid phase consisted of half MS basal medium supplemented with 0.50mg/l IBA and 2% sucrose which supports explants and is responsible for root induction. While, the liquid phase composed of MS basal salt supplemented with 2mg/l BAP, 0.25mg/l NAA and 3% sucrose responsible for shoot multiplication and growth. In case of DPS the semisolid phase consisting of rooting media was placed at the base of culture vessel supporting the explants, above which shooting media in liquid phase was poured. After 4 weeks liquid media (10ml/test tube) was added into the same culture vessel in sterile condition to maintain the culture [12]. In control experiment i.e. CMS; the explants were inoculated in semisolid media for shoot multiplication followed by rooting independently. All the cultures were incubated in culture room at 25±2 ^{0}C, 70% humidity and 3000 lux light intensity produced by white fluorescent tube light supplied by Saver Biotech Ltd, India. For each experiment 20 replicates were taken and each experiment was repeated thrice. Data (No. of multiple shoots, shoot length, No. of roots and root length) were taken in each 7 days interval and analyzed statistically by

the Duncan's multiple range test [13]. When well developed root system was observed plantlets were taken out from the culture tubes and washed carefully under running tap water for complete removal of agar media. Then the plants were transplanted to small polythene pots containing soil: sand: cow dung manure in 1:1:1 ratio. Then the pots were transferred to the green house for hardening. The plants were watered at every alternate day. The survival rate was recorded one month after the transfer into pots.

Cost analysis

A comparative analysis of the cost of the two different system i.e. CMS and DPS was done by taking three most important factors i.e. nutrient media, energy (electricity) consumption and labour cost. The cost of the nutrient media was calculated by multiplying the value of 1lt MS media = Rs 89.46 (by converting Kenyan shilling to rupees) [14] with the total amount media used for one complete experiment. The cost of energy consumption was calculated by the formula:

Cost of energy = [Total energy consumption X Unit cost of electricity] X Time taken for complete experiment (in month)

Total energy consumption = The total consumption of electricity towards lighting, cooling, air pumping and autoclaving [15] for one *in vitro* multiplication phase. Unit cost of electricity is 2.10/unit as per Chhattisgarh electricity board, India. The labour cost was calculate by the formula

Labour cost = Daily wages of skilled labour X Total time taken to complete the experiment

Daily wages of skilled labour for Chhattisgarh state as decided by Govt. of India is Rs 190/- (Minimum wages act, 1948)

Results

In a separate experiment maximum percentage of shoot bud proliferation (98%) was observed in MS + 0.5mg/l BA + 3% sucrose after 14 days of inoculation. Among the different media used for shoot multiplication and growth, the MS medium supplemented with 2mg/l BAP and 0.25mg/l NAA showed maximum percentage of shoot multiplication and shoot growth after 4 weak of culture. Half MS media supplemented with 0.5 mg/l IBA and 2% sucrose has given best rooting response in comparison to the other rooting media tested for *R. serpentina* (Table 2). These particular shooting and rooting medium was used both in DPS and CMS to compare the overall developments of the explants in both the system. As, DPS system consist of both shooting (liquid phase) and rooting media (semisolid phase) in the same culture vessel, explants were subjected to both rooting and shooting simultaneously from the very beginning of the culture. But in CMS, explants after proliferation, shoots were grown in semisolid shooting medium for shoot multiplication. Two subsequent sub cultures of the multiplied shoots were done at 4 weeks intervals in separate culture vessel followed by rooting in semisolid rooting media in separate culture vessel. It was found that the rate of shoot multiplication (98.7), shoot length (9.8) and shoot thickness (5.2) was higher in DPS (Table 3), but average number of multiple shoots (10.4) decreased when compared to CMS (Figure 1A). In DPS well developed roots were observed after 8 weeks of initial culture (Figure 1B) whereas in CMS it takes approximately 12 weeks (8 weeks for two subsequent subculture followed by 4 weeks for rooting) after initial culture up to root development. Moreover, the no. of roots/shoot (8.7), root length (5.3cm), root thickness (6.1mm) was found higher in DPS than that of the CMS (Table 3). Well developed plantlets with roots from both the systems were transferred to plastic pots for acclimatization and subsequently to field condition after one month. Acclimatization efficiency of about 98.5% was observed in DPS where as in control system it was 70% only. Well developed shoots with thick and large no. of roots in DPS may be the possible reason for better acclimatization (Figure 1C). The detail of the media used and their calculated cost is given in Table 3. The overall cost analysis result showed that in DPS cost of nutrient media, cost of energy consumption and labour cost has been reduced to 35.36%, 39.28% and 33.33% respectively in compare to the CMS (Table 4).

Parameters studied	Culture system	
	DPS †	CMS †
Percentage of shoot multiplication in the culture medium MS + 2.0mg/l BAP + 0.25mg/l NAA + 3% sucrose (mean ± SE)	98.7 ± 0.4 b	96.4 ± 0.8 a
Av. No. of multiple shoot/explants in the culture medium MS + 2.0mg/l BAP + 0.25mg/l NAA + 3% sucrose (mean ± SE)	10.4 ± 0.8 a	13.2 ± 0.7 b
Av. Shoot length (cm.) in the culture medium MS + 2.0mg/l BAP + 0.25mg/l NAA + 3% sucrose (mean ± SE)	9.8 ± 0.3 b	8.2 ± 0.5 a
Av. Shoot thickness (mm.) in the culture medium MS + 2.0mg/l BAP + 0.25mg/l NAA + 3% sucrose (mean ± SE)	5.2 ± 0.6 b	1.8 ± 0.2 a
Av. No. of roots/shoot in the culture medium ½ MS + 0.50mg/l IBA + 2% sucrose (mean ± SE)	8.7 ± 0.5 b	6.4 ± 0.7 a
Av. Root length (cm.) in the culture medium ½ MS + 0.50mg/l IBA + 2% sucrose (mean ± SE)	5.3 ± 0.8 b	4.9 ± 0.8 a
Av. Root thickness (mm.) in the culture medium ½ MS + 0.50mg/l IBA + 2% sucrose (mean ± SE)	6.1 ± 0.8 b	1.9 ± 0.5 a
Percentage of acclimatization efficiency(mean ± SE)	98.5 ± 0.7 b	78.3 ± 0.4 a

† 20 replicates per treatment, repeated three times Values are mean ± SE, * callusing at the basal end, means having same letter within rows were not significantly different at $P < 0.05$

Table 2: A comparative study on micropropagation of *Rauwolfia serpentine* in two different culture systems after 6 weeks of culture

Figure 1: Culture Showing in DM-DPS initiation (a), culture after 1 week (b), 2 week (c), 4 week (d), 6 week (e) and 8 week (f). Culture in CMS shown shoot multiplication after 4 week (g), culture in semi solid medium shown rooting after 4 week of inoculation in rooting medium (h). Acclimatization of dual- phasic culture plants after 45 days (i).

Name of the system	Name of different stages	Amount of different media used/1set of experiment#		Total cost of the media used in the experiment in different system
		MS	1/2MS	
CMS	Initiation	500 ml	---------	Rs156.56/-
	Multiplication*	1000ml	---------	
	Rooting	---------	500ml	
	Total amount of media used	1500ml	500ml	
DPS	Initiation	500ml	---------	Rs100.64/-
	Multiplication + Rooting	500ml†	250ml	
	Total amount of media used	1000ml	250ml	

* Two multiplication phase in each phase 500ml media was used

† 250ml used as liquid phase along with 250ml solid ½ MS and 250ml rest of the liquid media was used to maintain the culture

In each experiment 20 replicates were used

Table 3: Details amount of media used in different methods and their cost

Factors affecting micropropagation	Types of culture system		% of reduction in cost
	CMS	DPS	
Cost of Nutrient media/plant let (Total cost of media/ total no. of plants produced)	Rs 7.82/-	Rs 5.00/-	35.36 %
Energy cost /plant let	Rs 0.28/-	Rs 0.17/-	39.28 %
Labour cost/ complete experiment (initial to rooting)	Rs 15960/-	Rs 10640/-	33.33 %

Table 4: A comparison of overall production cost between CMS and DM-DPS

Discussion

MS medium supplemented with 2mg/l BAP and 0.25mg/l NAA showed maximum percentage of shoot multiplication and shoot growth after 4 weak of culture. Half MS media supplemented with 0.5 mg/l IBA and 2% sucrose has given best rooting response in comparison to the other rooting media tested for *R. serpentine*. Similar results were also reported by Salma et al. (2008) and Senapati et al. (2013) [8,11,16]. It was found that the rate of shoot multiplication (98.7), shoot length (9.8) and shoot thickness (5.2) was higher in DPS may be due to Increase contact surface area of explants with the media leads to an increase in diffusion, absorption and replacement of growth medium components that may have ultimately improved the growth responses in DPS. Whereas average number of multiple shoots (10.4) decreased when compared to CMS. The reduction in number of multiple shoots may be due to callusing at the base and increased shoot thickness. Similar results on shoot multiplication and growth were also reported by Pullman and Skryabina (2007) and Scherwinski et al. (2011) [5,4] in DPS where both the phase contains the same medium. In the dual phasic culture system as reported earlier they have shown the increased shoot multiplication and growth only at a time in the same culture vessel, where as the current system along with the increased shoot growth it has also shown a good rooting response in the same culture vessel. This is the first report where two different media were used in a single culture vessel. The no. of roots/shoot, root length and root thickness was found higher in DPS than that of the CMS this may be due to increase contact surface area of explants which ultimately increased the rate of diffusion/absorption. High percentage of Acclimatization was found in DPS than that that of CMS due to better growth of rooted shoots. In cost analysis a heavy reduction in propagation cost was observed in DPS than CMS as the repeated subculture was avoided in DPS. By this method the production cost of the plants were reduced and as these plants were *in vitro* raised these are disease free which is the sole objective of *in vitro* propagation. So that the farmer will able to get healthy disease free plant in low cost which ultimately reduced the farming cost of the farmer.

Adaptation of low cost tissue culture techniques by improving process efficiency and better utilization of resources without compromising the quality of the plants are of high priority for commercial utilization. The protocol described in DPS is a low cost technique saving 4 weeks of culture time. From this investigation one thing can be concluded that this protocol is very efficient in production of healthy and quality propagules of *R. Serpentine* in reduced time for commercial purposes. As this is a low cost and time saving method it has great potential for crop improvement using biotechnological approaches like genetic transformation and production of secondary metabolites.

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