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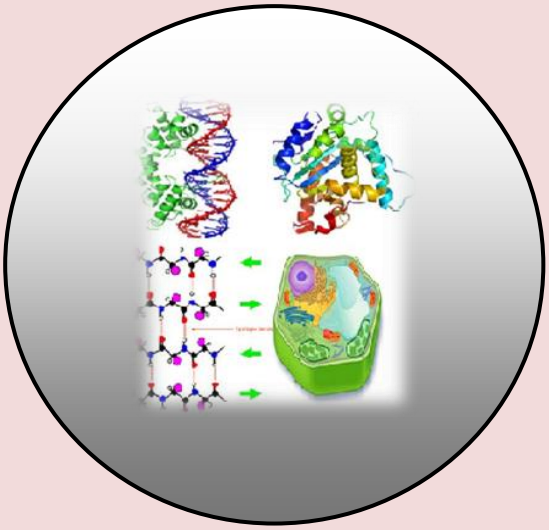
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

UGC Approved Journal No. 62923
MCI Validated Journal
Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 36 (1) 2019 Pages No. 116-121



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 36, No. 1: 116-121, 2019
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 36/01/8888/2019

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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RESEARCH PAPER

Received: 28/02/2019 Revised: 02/04/2019 Accepted: 03/04/2019

Development of Efficient Protocol for in vitro Mass Production of Seedlings of *Dendrobium sonia*: A Highly Important Ornamental Orchid

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ABSTRACT

Dendrobium Sonia is one of most popular orchid used extensively for cut flower production. Availability of planting material is the major challenge for the cultivation of the orchid. In this study efficient protocol develop for mass production of seedlings. Seeds were produced on healthy mother plants by hand pollination. Mature seeds after sterilization were inoculated on Murashige and Skoog's 1962 (MS) medium, on this, seed completed germination on 29.8 days of culture and rate of germination was 80.2%. Inclusions of plant growth regulators in MS medium significantly enhanced the germination process and seedling production. Among different growth regulator tested, BAP (0.5mg/l) along with NAA (0.5 mg/l) produced the most efficient response. On this medium 98.8% of the seeds germinated at 26 days culture and produced 5.6 number of leaves and 8.6 numbers of roots at 360 days of culture. Seedlings produced were successfully acclimated in the green house after transplanting into coconut husk pieces. The survival rates were of 90% with application of fertilizers and fungicides, the seedling produced new leaves and roots after 60 days of transfer.

Keywords: Pollination, Seed germination, In vitro, Growth regulators, Acclimatisation.

INTRODUCTION

Orchids belonging to the family Orchidaceae are highly valued for their extraordinary flowers qualities. The flowers are colourful; do have wide ranges of shapes and sizes; the self life also known to be very high. The family Orchidaceae is comprising of >26000 species under 800 number of genus (Chase et al., 2015). Using these, so far more than one lakh hybrids have been developed around the world and these hybrids are playing a major role in the floricultural industry. Among these, hybrids of *Dendrobium*, *Phalaenopsis*, *Cattleya*, *Oncidium*, *Cypripediums*, *Phragmipediums* and many more are the major players. In the cut flower industry, *Dendrobium* hybrids are dominant varieties, being used extensively in many part of the world. Because of the bright colours and long shelf life, demands of these varieties are growing sharply day by day. Again among the *Dendrobium*s, the Sonia varieties are highly valued for the cut flower production. The Sonia varieties are developed using the *Dendrobium* Caesar and *Dendrobium* Tomie Drake as the parents. In the market, currently there are 6 (Sonia 17, Sonia 19, Sonia 28, Emma White, Sakura Pink, Erkasul) numbers of Sonia varieties are available and many of these are extensively used for the commercial cultivation particularly for cut flower production.

The flowers do have mix colour of bright pink and white colours, shape of flower are quite attractive. Inflorescence is of 45-50 cm in length and the healthy plants do produce in an average of 20 flowers. Shelf life of the flowers is of 8-9 weeks on plants and 15-20 days as cut flowers. In cultivation each plant will produce 6-8 inflorescences each year. The major bottleneck of this cultivation is the availability of planting materials.

Orchids in general under natural conditions propagate through seeds as well as by vegetative means. These members are producing seeds in thousands; however, under natural condition require the symbiotic association of fungus for germination and subsequent production of seedlings. The seed germination rate is extremely low, in majority cases confined to less than 5%. Again this process requires ideal microclimate condition, difficult to establish under ordinary laboratory conditions. Propagation through vegetative means involves production of keikis, offshoots and separation of pseudobulbs; the process is extremely slow and only few plants could be produced in a year. With the development of biotechnological tools like, development of Knudson C medium (1946) and production of plantlets through meristem culture (Morel, 1960), production of planting materials for orchids are becoming much easier and simpler. Both the seeds as well as the meristems of orchid could be cultured under in vitro conditions; with use of appropriate growth regulators, thousands of the plants could be produced (Teixeira da Silva et al. 2015; Shen et al. 2018).

In this study, highly efficient protocols were developed for the mass production of planting materials of *Dendrobium* Sonia orchid. The seeds are used as the explants and cultured on nutrient medium. The effect of different growth regulators on seed germination as well as on further growth and development were studied.

MATERIALS AND METHODS

The *Dendrobium* Sonia plants growing at the orchidarium of the institute were used for collection of seed bearing capsules. For production of seeds, pollinations were made on healthy flowers by hand and were bagged in order to avoid cross pollinations. This practice was required for the production of genetically pure seeds. After pollination, seeds were allowed to mature for 2 - 2.5 months. At this stage it was found that the seeds had well developed embryo surrounded by a seed coat.

The mature seed bearing capsules were brought to the laboratory, washed thoroughly under running tap water and then brought to laminar air flow cabinet for surface sterilization. For this purpose, 0.1 % solution of Sodium Hypochlorite was used for2 minutes followed by treatment with 70 % ethanol for 1 minute. Finally, capsules were washed with sterile distilled water for removal of sterilant from the surface. Murashige and Sookg, 1962 (MS) medium was used for germination and subsequent seedling development of the orchid. The pH of the medium was maintained at 5.8 and medium was semi-solidified with use of 6 % Agar (HiMedia, India). Mediums were sterilized at 120^{o}C and 15 Lb pressure for 20 minutes. After sterilization, the mediums were kept for three days in the media preparation room. Surface sterilized capsules were cut and seeds were dusted on mediums. Then cultures were kept in culture room racks where temperature is maintained at 25± 2°C and 75 % relative humidity under cool fluorescent light of 40 µmol m^{-2} s^{-1} (Philips, India) with a 16h photoperiod.

Effects of the growth regulators on seed germination and seedling development were studied. For these experiments MS medium was supplemented with 0.25, 0.5, 1.0, 1.5 mg/l 6-Benzylaminopurine (BAP), 0.5, 1.0, 2.0 mg/l Kinetin (Kn) and 0.5, 1.0, 2.0 mg/l 1-Naphthaleneacetic acid (NAA). In addition combinations of BAP and NAA (0.25 + 0.5, 0.25 + 1.0, 0.25 + 1.5, 0.5+ 0.5, 0.5+1.0, 0.5 + 1.5 mg/l; BAP flowed by NAA) and combinations of BAP and IBA (Indole-3-butyric acid) (0.25 + 1.0, 0.25+ 2.0, 0.5+1.0 0.5 + 1.0 and 0.5 + 2.0 mg/l, BAP followed by IBA) were also studied. The growth regulators were added before adjustment of pH of mediums and latter sterilised as mentioned above. Calculation of seed germination rates were made after 30 days of culture and number of roots and leaves were calculated after 360 days of culture. For each treatment, data recorded from five numbers of replications and mean and standard error of the mean were calculated. The statistical analysis carried out by using Duncan's multiple range test (DMRT).

Experiments were carried out for efficient induction of root on the plant produced from different culture mediums.

The plant were removed from the culture bottles and transferred to MS medium containing different concentrations (1.0, 2.0 & 3.0 mg/l) of NAA and IBA. Inductions of roots were observed at 90 days of culture. For each treatment five numbers of explants were used. Different statistical parameters were measured such as mean and standard error of mean. The relationship between the means was established using DMRT.

Well developed seedlings were removed from culture bottles, washed thoroughly under running tap water to remove agar. These were then transplanted into coconut husk pieces and kept in green house for acclimatization. Fertilizers (N19:P19: K19) 1 gm/l and fungicide (bavistin) 1 gm/l applied on plants twice a week for proper growth and development.

RESULTS AND DISCUSSION

In this study efficient protocols were developed for the production of healthy seedlings. Mature seeds were used and found to be highly active, majority of them were able to germinate on nutrient medium. Dormancy of the seeds has been reported in many orchids, particularly in terrestrial orchids (Godo et al. 2010). In many of them, mature seeds are dormant, require special treatments for germination. In case of *Dendrobium* Sonia, the seed seems to not associate with dormancy. MS medium used in the study, on which seeds swelled fast after seven days of culture. The embryos turned green after 15 days of culture and majority of them completed germination at 29.8 days. The rate of germination on medium without any supplements was 80.2%, seems that the small seeds acquired all required chemicals for germination. On MS medium seedlings were produced and at 360 days of culture produced 3 numbers of roots and 2.4 number of leaves in each seedlings. Earlier Kundson C, 1946 and Vacin and Went, 1949 were used in number of orchids (Zhang et al. 2015).These were simpler in combination as compared to MS which is rich in different components like nitrogen, vitamins, and amino acid and carbon sources. In recent years, numbers of successful studies were carried out using MS medium particularly in epiphytic *Dendrobium* orchids (Mohanty et al. 2012). In a study carried out on *Paphiopedilum armeniacum* that used three different medium such as MS, Kundson C and Vacin and Went, among this, MS medium shown to the best medium for seed germination (Zhang et al. 2015).

In order to develop an efficient protocol, growth regulators were added to the MS medium. The effects of growth regulators on seed germination and seedling development are mentioned in Table 1. It was found that the seed germination rate was enhanced with the supplement of BAP irrespective of concentration used. The seeds also completed germination in Kn containing mediums. With the lower concentration of BAP (0.25 mg/l), the seeds completed germination at 25.2 days of culture and rate increased significantly to 99.4% with the increase of concentrations of BAP, the effects were similar to all other concentrations used in the study (Table 1).In analysing the effects on seedling production, it was found that with addition of BAP completely inhibited root production. Similar kinds of enhancing seed germination effects have been reported in orchids. (Chung et al. 2005; Kong et al. 2007). Inclusion of Kn at lower concentrations (0.5 and 1.0 mg/l) did not enhanced the seed germination rate as compared to the control, however, with 2.0 mg/l Kn enhanced the germination rate. Addition of NAA Individually in the medium though did not enhanced rate of germination, produced more number of leaves and roots. Combinations of cytokinin and auxins were also tested, it was found that BAP and NAA combinations produced more leaves whereas BAP and IBA combinations produced more roots. Among the six combinations of BAP and NAA test, the combinations of 0.5 mg/l NAA+ 0.5 mg/l BAP produced the best seed germination of 98.8%, seeds completed germination after 26 days of culture, produced protocorm like bodies (PLBs) after 60 days of culture (Fig. 1a). The plant attained he height of 3 cm with well-developed leaves after 180 days (Fig. 1b). In this medium 5.6 number of leaves and 8.6 numbers of roots produced at 360 days of culture (Fig. 1 c). This medium found to be most efficient medium for seedling production in *Dendrobium* Sonia orchid. Inclusion of BAP and IBA in the nutrient medium, in two combination (0.5 + 1.0 and 0.5+ 2.0 mg/l BAP followed by IBA) not only enhanced the germination process to 84.6% and produced more leaves and roots. It is found that in many orchids inclusion of growth regulators plays major role not only for seed germination but also for seedling development; however, the exact requirement varies from species to species (Hossain et al. 2010, Abraham et al. 2012).

Table 1. Effect of growth regulators on seed germination and seedling development in vitro.

Growth regulators (mg/l)	Days taken for germination	% of germination[a]	No. of leaves/ shoot[b]	No. of roots/ shoot[c]
MS (Control)	29.8 ± 0.48 a	80.2 ± 0.86 ef	2.4 ± 0.24 i	3.0 ± 0.00
BAP				
0.25	25.2 ± 0.37 ij	99.4 ± 0.60 a	3.6 ± 0.24 gh	0.0 ± 0.00 h
0.5	25.0 ± 0.31 j	99.8 ± 0.20 a	3.0 ± 0.00 hi	0.0 ± 0.00 h
1.0	25.4 ± 0.40 hij	98.6 ± 0.24 a	3.8 ±0.20 fgh	0.0 ± 0.00 h
1.5	25.8 ± 0.20 ghij	98.4 ± 0.24 a	4.6 ± 0.40 def	0.0 ± 0.00 h
Kn				
0.5	26.2 ± 0.37 fghi	80.6 ± 0.40 ef	3.4 ± 0.4.0 gh	0.0 ± 0.00 h
1.0	26.4 ± 0.60 efgh	80.4 ± 0.40 ef	3.4 ± 0.24 gh	0.0 ± 0.00 h
2.0	26.8 ± 0.37 defg	84.4 ± 1.4 6d	4.2 ± 0.20 efg	0.0 ± 0.00 h
NAA				
0.5	26.4 ± 0.24 efgh	81.4 ± 1.40 de	3.2 ± 0.48 hi	5.2 ± 0.37 ef
1.0	27.0 ± 0.00 def	80.2 ± 0.58 ef	3.4 ± 0.24 gh	6.6 ± 0.40 d
2.0	27.2 ± 0.37 cde	78.8 ± 0.37 ef	3.8± 0.37 fgh	7.2 ± 0.37 d
BAP + NAA				
0.25 + 0.5	27.0 ± 0.00 def	93.8 ± 0.37 b	4.6 ± 0.24 def	4.4 ± 0.40 f
0.25 + 1.0	27.6 ± 0.24 cd	92.4 ± 0.67 bc	5.4±0.40 abcd	6.2 ± 0.20 de
0.25 +1.5	28.0 ± 0.54 bc	90.0 ± 1.41 c	5.2±0.37 abcd	6.6± 0.24 d
0.5 + 0.5	26.0 ± 0.00 fghij	98.8 ± 0.20 a	5.6± 0.24 abc	8.6 ± 0.60 c
0.5 + 1.0	26.2± 0.20 efghi	94.6 ± 1.20 b	6.0 ± 0.00 a	8.8 ± 0.48 c
0.5 + 1.5	26.2± 0.20 efghi	93.6 ± 1.16 b	5.8 ± 0.20 ab	9.0 ± 0.63 bc
BAP + IBA				
0.25 +1.0	28.6 ± 0.24 b	78.2 ± 0.58 ef	4.8± 0.20 cde	9.2 ± 0.20 abc
0.25 + 2.0	28.8 ± 0.37 ab	78.0 ± 0.44 f	5.0±0.31 bcde	9.6 ± 0.24 abc
0.5 + 1.0	26.4 ± 0.24 efgh	84.6 ± 1.50 d	5.2±0.20 abcd	9.8 ± 0.37 ab
0.5 + 2.0	26.6 ± 0.24 defg	84.4 ± 2.42 d	5.4±0.24 abcd	10.0 ± 0.31 a

[a] Seed germination rates were observed at 30 days of culture,
[b]&[c] Number of leaves and roots per plant were recorded in 360 days of culture

Table 2. Effects of growth regulators on root production.

Growth regulators (mg/l)	No. of leaves/ shoot[a]	No. of roots/ shoot[b]
MS	2.6 ± 0.24 g	2.8 ± 0.37 f
IBA		
1.0	5.4 ± 0.24 c	8.8 ± 0.37 c
2.0	6.4 ± 0.24 a	10.4 ± 0.24 a
3.0	5.0 ± 0.24 e	8.6 ± 0.24 d
NAA		
1.0	5.2 ± 0.20 d	8.6 ± 0.24 d
2.0	6.2 ± 0.20 b	10.0± 0.31 b
3.0	4.8 ± 0.20 f	8.4 ± 0.24 e

[a]&[b] Number of leaves and roots per plant were recorded in 90 days of culture

In a study carried out in *Comparettia falcata* by Manirique et al. (2005) inclusion of Kn increased the seed germination. Similarly, addition of BAP 1.0 mg/l enhanced the seed germination in *Epidendrum ibaguense* (Hossain, 2008). It is also reported that the combined effects of growth regulators (BAP and NAA) benefited number of orchids in producing healthy seedlings (Diengdoh et al. 2017)

The plants produced without roots were transferred to MS medium containing different concentrations of NAA and IBA as mentioned in Table 2. Inclusion of both regulators enhanced root induction process. Among the two, there is no difference in response. In both growth regulators 2.0 mg/l found to be best concentrations for root induction, at 90 days of culture about 10.4numbers of roots produced in each seedling. The roots have attained 3-4 cm in length and have acquired 6.4 numbers of leaves (Table 2).

Healthy seedlings were transferred to coconut husk pieces and kept in the green house for acclimatization. The survival rates were 99%, after 60 days of acclimatization seedlings produced new leaves and roots (Fig. 1d). These were then transferred to orchid pots for further growth and development (Fig. 1 e).

Figure 1. Different stages of seedling development of Dendrobium Sonia under in vitro condition on MS+ BAP 0.5 mg/l+ NAA 0.5 mg/l.

a. PLBs formation from the seeds after 60 days of culture
b. Leaf production from the PLBs after 180 days of culture
c. Seedlings with well developed leaves and roots after 360 days of culture
d. Acclimated seedlings on coconut husk pieces
e. Seedling transplanted to orchid pots

CONCLUSION

Highly efficient protocol developed for the production of seedlings required for cut flower production. Seed were produced by hand pollination and mature seeds collected. On inoculation on MS medium supplemented with 0.5 mg/l BAP along with 0.5 mg/l NAA, 98.8 % of seed germinated at 26 days of culture. On this medium each seedling produced 5.6. number of leaves and 8.6 number of roots at 120 days of culture. Whenever required roots were efficiently induced with the addition of 2.0 mg/l IBA. The survival rates of seedlings in the green house were 90%.

ACKNOWLEDGEMENTS

The authors are highly grateful to the Chief Executive of Regional Plant Resource Centre for support.

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