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Research methodology is one of the most important revolutionary subject with tremendous impact on the welfare of mankind in terms of doing contemporary research. The potentials are enormous and many breakthroughs are being observed day by day with respect to healthcare, food, agricultures and environmental protection which are being covered in this subject. Moreover, it is a fast growing field in the world and done remarkable achievements for human mankind in doing latest research using various tools and techniques.

Due to the impact of COVID-19 pandemics and serious outbreak across the world, there is a transform happening among Educational Institutions and Industries to teach the contents through online mode. In this context, webinars and online mode teaching are playing important role to manage the time bound activities. It is intended to impart the knowledge on creating various research methodologies to the stakeholders.

Various topics such as how to write project proposals and research papers, higher Education in India and abroad, concept of Moocs, Moodle, Swayam in teaching, role of fitness & wellness care on cognitive skills of researchers, types of research and sponsored scheme, experimental planning and execution, review of literature and resources, presentation and interpretation of results, effective presentation skills, types of scientific and technical publications, importance of impact factor and citation index, plagiarism tools and their role in publications, dissertation, thesis and project report writing, instrumentation tools and tecshniques, biochemical calculations and matrics, statistical tools in research, ICT tools and Bioinformatics in research, IPR and Biosafety, teaching methodologies and pedagogies and importance of Google labs, Google tools and E-Library were discussed in brief.

We owe our special thanks to Mrs. S. Rajalakshmi Jayaseelan, Chairman, Dr. B. Mythili Gnanamangai, Vice-Chairman, Dr. P.V. Sreenivasan, Director and Mrs. S. Priyadharsini, Assistant Director, Nature Science Foundation, Coimbatore, Tamil Nadu for their whole hearted support and tireless efforts to edit this book in a grand manner. We profusely thank Dr. S. Gopalakrishnan, Principal, KSR College of Technology, Tiruchengode, Tamil Nadu for his constant support and encouragement in

editing this book neatly. We also thank all the members for scrutinizing and fine-tuning of the manuscripts.

We appreciate all the authors of the book for their excellent efforts in writing their book chapters in a neat form in the journey of multidisciplinary subject in the world. There is no doubt that this book definitely useful for all the researchers in their research journey.

The words are inadequate to express the sincere thanks to M/s. AkiNik Publishers, New Delhi for preparation of the book very neatly and taking pains in bringing out the book very successfully to the delegates and participants on time. We hope the book will be of more useful for readers and researchers too.

P. Ponmurugan

J. Philip Robinson

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Content

Chapter - 10
Assessment of Plant Growth Regulators on Micropropagation of *Pedalium murex* l. – A Valuable Medicinal Plant

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Chapter - 10

Assessment of Plant Growth Regulators on Micropropagation of *Pedalium murex* L. – A Valuable Medicinal Plant

Dr. K. Ganesh Kumari

Abstract

An efficient reproducible *in vitro* protocol for *Pedalium murex* L. was developed. For multiple shoot formation, Murashige and Skoog (MS) medium with B5 Vitamins was employed for the direct multiplication of shoot tip explants. Different Plant growth regulators like 6-Benzyl amino purine (BAP) (4.44 – 13.32 µM), Kinetin (KIN) (2.32 – 13.92 µM), Indole-3-acetic acid (IAA) (0.28 – 1.43 µM), Indole-3-butyric acid (IBA) (0.24 – 1.71 µM), 1- Naphthalene acetic acid (NAA) (0.26 – 1.34µM), Adenine sulphate (AdS) (0.144 – 0.867 µM) with carbohydrate sucrose and Maltose (10 – 50g/l) along with some additives like Activated charcoal (AC) (0.5 – 3.0 mg/l), Polyvinylpyrrolidone (PVP) (10 – 50 mg/l), Ascorbic acid (ASC) (10 – 50mg/l) and Citric acid (CA) (10 – 40mg/l)were used individually and in combinations for efficient multiplication of shoots. Among the different concentrations and combinations tested, shoot ip explants produced maximum number of shoots (23.8 shoots/explant) on the media comprising of MS salts, B5 vitamins, BAP (8.88µM), IAA (0.57µM) and AdS (0.289µM). Among various concentrations of sucrose and maltose tested, sucrose 30g/l showed the best response with 97.6% of shoots / shoot tip explant. Addition of additives particularly PVP (20mg/l) control the phenolic secretion and induced 10.9 roots form the elongated shoots. The rooted plants were hardened on plastic cups and kept on environmental growth chamber.

Keywords: *Pedalium murex*, *in vitro* culture, plant growth regulators, additives, hardening

Introduction

Medicinal plants have been used in virtually all cultures for the treatment of different diseases. The use of herbal remedies and healthcare

preparations, as those described in traditional methods, are obtained from commonly used medicinal plants, has been traced to the occurrence of natural products with medicinal properties. The use of traditional herbal medicine in most developing countries has been widely observed (UNESCO, 1996). Compared with India, the practice of traditional medicine is widespread in China, Japan, Pakistan, Srilanka and Thailand. At the same time, the cultivation of endangered medicinal plants species in agriculture land becomes difficult due to several problems. Loss of seed formation, loss of seed viability, unfavorable situation for seed germination and damages caused by insect pests etc were considered as important factors controlling the seed germination of economically important medicinal plants. Hence, the studies on the regeneration and large multiplication of endangered plant species through available methods become essential.

Based on the literatures available, we found that *Pedalium murex* L. belongs to the family Pedaliaceae is considered as one of the traditional medicines used for multipurpose treatment in healthcare science. The roots, leaves as well as young and mature seeds were used as medicines (Sundaram et al., 1999; Rajendra et al., 1992; Subramanian and Nair, 1972). The seeds grow only during rainy session and hence, the seed availability during winter and summer become very difficult. Hence, we planned to expose the *P.murex* in to tissue culture techniques for large scale multiplication. If any plant species introduced into *in vitro* regeneration techniques, it is possible to regenerate the plantlets in any session without any difficulties. Based on these, we planned to regenerate the *P.murex* through tissue culture techniques.

Regarding *P.murex in vitro* regeneration, very few regeneration protocols has previously been reported. Direct multiplication, callus induction and callus mediated whole plant regeneration are the major ways for whole plant regeneration. Unfortunately, due to several regeneration problems, regeneration of *P. murex* is recalcitrant. Hence, there is an urgent need to standardize the regenerative protocol.Hence, this research work was targeted to achieve a simple and effective reproducible protocol for the regeneration of *P. murex* through direct multiplication by shoot tip and node explants.

Materials and Methods

Plant material and seed germination

Pedalium murex shoot tip explants were collected from the Botanical garden of Bharathidasan University, Trichy, Tamil Nadu, India. The

collected explants were surface sterilized by using 4 drops of teepol (soap solution) for one minute followed by kept under running tap water for 1 hour to remove the soap solution. After one hour, inside the laminar air flow the explants were treated with 70% alcohol for 1 minute and 0.1% of $HgCl_2$ for 3 minutes. The explants were washed with sterile distilled water for 3 – 5 times to remove the further sterilant. The inoculated tubes were transferred to light intensity (15u mol m^{-2} s^{-1}), 16 h light / days photoperiod at 25 ± 2ºC and 55-60% relative humidity.

Multiple shoots induction

MS (Murashige and Skoog, 1962) basal medium with B5 vitamins (Gamborg et al., 1968) was employed for the direct multiplication of explants. Shoot tips with one or two leaf primordial were taken form 12 days old seedlingsfor direct shoot multiplication. The shoot tips of 5-6 mm in length were excised approximately. Sucrose (3%) was used as carbohydrate source in all the media. Growth hormones such as, Benzylaminopurine (BAP) (4.44 – 13.32µM), Kinetin (2.32 – 13.92µM), Indole acetic acid (IAA) (0.28 – 1.43µM), Indole butyric acid (IBA) (0.24 – 1.71µM), naphthalene acetic acid (NAA) (0.26 – 1.34µM) and Adenine sulphate (AdS) (0.144-0.867µM) were tested individually as well as in combinations at different concentrations to the medium for multiple shoot induction. (Graph 1).

The prepared medium was autoclaved and placed in dark condition upto inoculation. After the inoculation of explants multiple shoot induction was observed. The induced multiple shoots were further sub cultured in the same medium for shoot elongation. During the process of multiple shoot induction, 8-10 days subculture was strictly followed. Otherwise browning of explants on medium was noticed. During each experiment 250 explants were taken for multiple shoot induction and each experiment was repeated three times.

Effect of carbohydrates on shoot multiplication

The effect of different carbohydrates (sucrose and maltose) 10-50 g/l were supplemented individually as well as in combination to study shoot multiplication (Graph 2).

Rooting and Hardening

Well-developed shoots (1.5cm and above in length) were excised from the culture tube and sub cultured onto the medium containing MS salts supplemented with IAA (2.46-12.3uM), IBA (2.85-14.26 uM) and NAA

(2.69-13.43uM) (Graph 3) and additives [(activated charcoal (AC, 0.5-3.0 g/l), polyvinyl pyrrolidone (PVP, 10-50 mg/l), ascorbic acid (ASC, 10-50 mg/l) and citric acid (CA, 10-40 mg/l)] to study root induction (Graph 4). The cultures were kept under dark for 48 hours and then transferred to light intensity (15 µ mol m^{-2} s^{-1}), 16 hr light / day photoperiod. After 2 weeks, the rooted plantlets were removed from the culture tubes and washed in the running tap water. The number of roots in each plantlet was counted and they were transplanted into plastic pots containing sterile sand, soil and farmyard mixture (1:1:1). The pots were covered with transparent polythene bags to maintain humidity under 16 hour light / day photoperiod at light intensity (15 µ mol m^{-2} s^{-1}) at 25 ± 2C. The plants were nourished using half strength MS nutrient solution as and when required. The well-grown plants were transferred to larger pots containing soil mixture and placed in greenhouse. Later the plants were transferred to field.

Statistical analysis

Mean and standard error were used throughout the study and the values were assessed by using a parametric moods median test (Snedecor and Cochran, 1989). For multiple shoot induction from shoot tip explants, 30 explants were tested with 5 replicates and each experiment was repeated three times. During root induction, 30 elongated shoots were tested for each treatment and each experiment was repeated 3 times with 5 replications.

Results and Discussion

Multiple shoot induction from shoot tip explants

Effect of plant growth regulators

For direct multiple shoot induction, the impact of different plant growth regulators like, BAP, Kin, BAP in combination with IAA, IBA and NAA and BAP in combination with NAA and AdS were tested. In all the treatments of plant growth regulators, multiple shoot induction was noticed from the explants. Among the different treatments, medium comprising of MS salts, B5 vitamins, BAP (8.88 uM), NAA (0.57 uM) and AdS (0.289 uM) showed best response for multiple shoot induction. In this concentration, maximum of 23.8 shoots were regenerated from single shoot tip explant (Graph 1) (Figure 1). Individual treatment of cytokinin and combined treatment of cytokinin along with small amount of auxins showed low percentage of multiple shoot induction when compared with above concentration. When compared with BAP and Kin, BAP showed superior percentage of response for multiple shoot induction. Hence, for combined treatment studies, along with auxins, BAP was selected. In combined

treatments, supplementation of IAA (0.57 uM) and IBA (0.74 uM) along with BAP (8.88 uM) showed high response when compared with BAP and NAA treatment.

Our result recorded multiple shoot induction of *P. murex* in lower concentrations compared to the report of (Ramar and Nandagopalan, 2011)in which the shoot tip explants produced multiple shoots on BAP (4 mg/l) and in combination with IAA (2 mg/l). Ravinder Singh and Kathiresan (2013) During direct multiple shoot induction, the type of auxin supplementation showed variation for multiple shoot induction. The combination of BAP, AdS along with IAA showed superior response. Like our results, this type of auxin dependent response for direct multiplication was noticed in several dicot plants like *Lippia junelliana* (Julani et al., 1999) and in Peanut (Bhanumathi, 2005).Apical and axillary bud based multiplication methods have been found successful in several dicot plants likePetunia (Ulian et al., 1988), Pea (Hussey et al., 1989), Sunflower (Bidney et al., 1992; Schrammeijer et al., 1990), Banana (May et al., 1995), Tobacco (Zimmerman and Scorza, 1996), Rice (Park et al., 1996), and in Cotton (Ganesan and Jayabalan, 2005). In some cases, combinations of BAP or Kin with any auxins were used for effective multiple shoot induction in *Hedeoma multiflorum* (Koroch et al., 1997). Like our studies, BAP-mediated plant multiplication and regeneration was noticed along with auxins and AdS. BAP found to be ideal hormone for shoot multiplication of grain legumes by using shoot tip explants (Kartha et al., 1981). Overall, these results proved that BAP found to be more effective for direct multiple shoot induction from meristem-based explants.

Effect of Ads

The frequency of multiple shoot induction was increased after the addition of Ads along with cytokinin (BAP – 8.88 uM) and auxin (IAA – 0.289 uM). Among the various concentrations, supplementation of 0.289 uM of AdS produced high multiple shoot induction frequency. Individual supplementation of AdS to the medium was not effective, but combination of BAP, IAA and AdS influences shoot formation. AdS promoted maximum shoots in *Macrotyloma uniflorum* (Varisai Mohamed et al., 2004) during direct regeneration. According to Gulati and Jaiwal (1992, 1994) in *Vigna radiata* AdS ($5x10^{-6}$ M) produced, maximum number of shoots per explant and also suggested that addition of different concentrations of AdS considerably induced multiple shoot formation (Graph 1)

After the induction of multiple shoots, the shoots clusters were sub-cultured on the same mother media. At the end of fourth week, the induced shoots reached maximum height of 10.6 cm per shoot. This result proved that there is no need of addition of any new plant growth regulator for shoot elongation. Usually during shoot elongation, the isolated single shoot lets or shoot clusters were transferred to shoot elongation medium containing different concentration of BAP and / or GA_3 (Xie and Hong, 2001), *Lonicera tatarica* (Palachlos et al., 2002) and *Solanum melongena* L. (Magioli et al., 1998). But our experiments proved that, there is no requirement of separate medium or plant growth regulator for shoot elongation.

Impact of carbon sources on multiple shoot induction

The influences of different carbohydrates were also evaluated for the induction of high frequency of multiple shoots. Individual and combined effects of sucrose and maltose were tested for this present investigation. Among various concentrations of sucrose and maltose tested, sucrose 30 g/l showed the best response with 97.6% in shoot tip with high frequency of multiple shoot induction. Individual treatment of maltose and combine treatment of maltose with sucrose showed very low response for the direct induction of multiple shoots from the explants. At the same time, high frequency of basal callus formation and abnormalities induction were observed in the above concentrations (Graph 2; Figure 1).

Usually direct multiple shoot induction and plant regeneration was achieved by the supplementation of sucrose as carbon source (Radhakrishnan et al., 2001). Like this report, in our studies also, high frequency of multiple shoot induction was obtained on the media fortified with sucrose. In nature, sucrose is transported within plant tissues and plants have an inherent capacity for uptake, transport and utilization of sucrose (Eapen and George, 1993). Sucrose was superior to maltose in promoting shoot growth, because organogenesis is a high energy requiring process. Like our observations, instead of sucrose, addition of maltose showed poor response for multiplication of several dicot plants including peanut (Mhatre et al., 1985 and Bhatia et al., 1985).

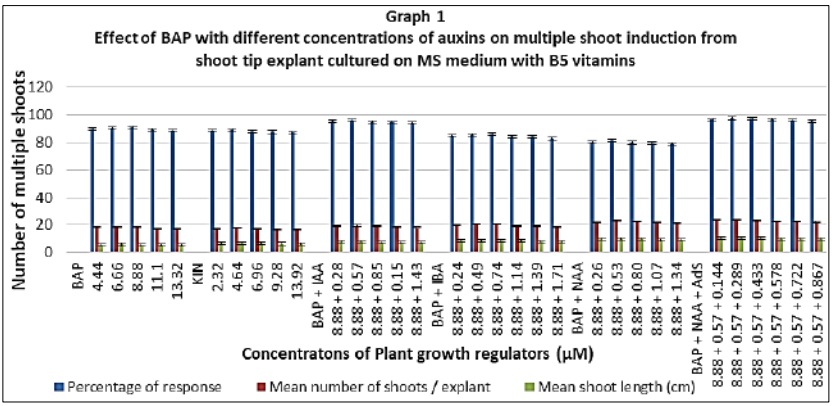
Root induction and hardening

For root induction studies, the elongated multiple shoots from the shoot clusters were individually separated and were kept for rooting using MS supplemented with various auxins. Initially, for root induction supplementation of all the auxins tested (different concentrations of IAA, IBA and NAA) showed poor response. Instead of root formation, basal

callus was noticed. Hence, we reduced the medium strength up to half. After the reduction of medium strength, we found root induction from the elongated shoots cultured on medium comprising half strength MS salts, B5 vitamins and IBA (5.71 uM) (Data not shown). *In vitro* root induction is usually difficult in several medicinal plants due to high frequency of basal callus formation and phenolic oxidation process (Soniya and Das, 2002). In our studies also the same difficulties were observed. Hence, we tested different additives to control the phenolic exudation process. After two weeks, we found that the percentage of root induction was accelerated by the addition of additives particularly PVP. This result proved that addition of additives can control the phenolic secretion from the explants and simultaneously enhanced the rooting percentage (Graph 3; Figure 1). In other dicot plants, like cotton, supplementation of charcoal along with media showed the best response for root induction *in vitro* (Trolinder and Shang, 1991; and Zapata et al., 1999). The root induced shoots were transferred to plastic pots for hardening. Survival of about 98% *in vitro* derived plants was observed after 1 month. The fully regenerated plantlets under greenhouse condition were transferred to field for further development.

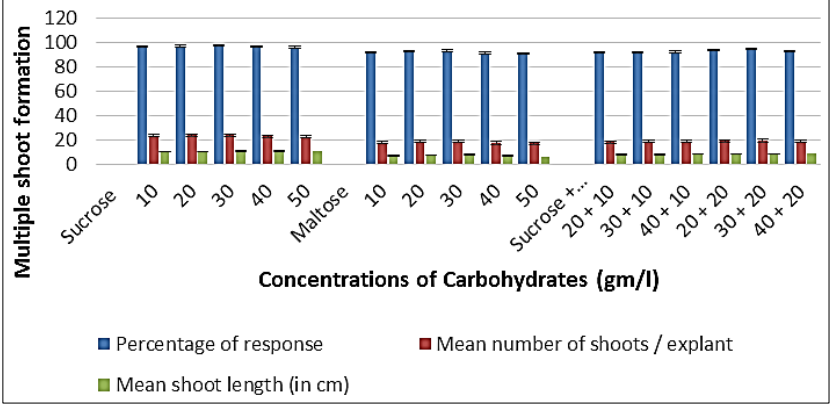
Conclusion

In conclusion, an efficient and simple protocol for *in vitro* adventitious shoot multiplication from callus cultures and whole plant regeneration has been described. The protocol was optimized by manipulations of different PGRs, carbohydrates and additives for enhanced multiplication. The protocol described in this research paper provides a rapid plant regeneration from shoot tip explant for *Pedalium murex* which can be used for the future research like somaclonal variation and producing transgenic plants in *Pedalium murex* through Agrobacterium and biolistic methods.



Graph 2

Individual and combined effect of various concentrations of sucrose and maltose on multiple shoot induction of shoot ip explant cultured on MS medium fortified with B5 vitamins and different concentrations of BAP (8.88µM), AdS (10.88µM) and GA3 (0.



Graph 3

Effect of different additives on root induction of elongated shoots cultured on medium fortified with MS salts, B5 vitamins and IBA (5.71µM)

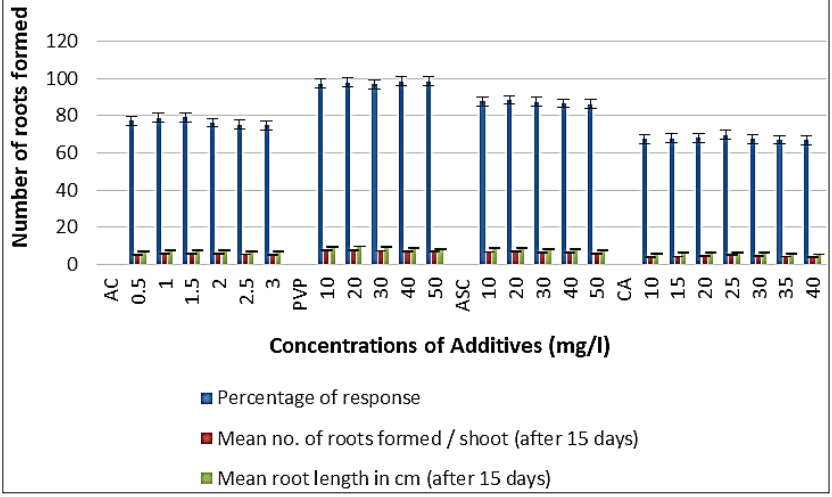


Fig 1: Multiple shoot induction from shoo tip explant a) Shoot tip explant after seven days of inoculation (Bar – 0.5cm). b) Initiation of adventitious multiple shoots after 15 days (Bar – 0.5cm). c) Elongated multiple shoots after 25 days (Bar – 0.5cm). d, e & f) Rooted shoots with flower heads (Bar – 0.5cm). g) Hardened plantlet in plastic pots (Bar – 0.75cm). h) Hardened plants grown in environmental chamber (Bar – 4.0 cm)

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