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Optimization of Protoplast Isolation and Micropropagation Techniques in Rose

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ABSTRACT

Rose is the dominant flower in the ornamental industry because of its multiple aesthetic values. Optimization of plant growth regulators in media was achieved for one of the important species of rose. The nodal explants were tested on a full strength MS media with different hormone concentrations of 6-benzylaminopurine (BAP) and Kinetin (Kn). Maximum response was obtained for a combined hormone concentration of 6-benzylaminopurine (3.5mg/L) and kinetin (1.5mg/L) with shoot regeneration from nodes, internodes and apical meristematic tissue. 6-benzylaminopurine alone showed shoot regeneration only from internodes and nodes, while kinetin alone showed shoot regeneration only from apical meristematic tissue. Protoplast of *Rosa sp.* and *Rosa sinensis* were isolated enzymatically and fused using polyethylene. The protoplasts were observed to fuse on different planes in an irregular pattern.

Key words: Micropropagation technique, Polyethylene glycol, Protoplast fusion, Rosa sp., Rosa sinensis.

Abbreviations used: PGR- Plant Growth Regulator, BAP- 6-benzylaminopurine, Kn- Kinetin, PVP- Polyvinyl pyrollidone, PEG- Polyethylene glycol.

INTRODUCTION

Rose is an important commercial crop having very high demand in the perfumery and cosmetic industries. Fossils of rose that was found in Oregon and Colorado (USA) were estimated to be more than thirty five million years old (Fairbrother 1965). Some ancient civilizations of China, western Asia and northern Africa (Shepherd 1954) were known to have cultivated rose as tombs had rose fossils. Rose is a symbol for secrecy,virtue, womanhood etc. Out of the 120 or more species of the genus *Rosa*, only eight of the species- *R. chinensis*, *R. damascena*, *R. foetida*, *R. gallica*, *R. gigantea*, *R. moschata*, *R. multiflora* and *R. wichuriana*of the sections in Indicae, Gallicanae, Pimpinellifoliae and Synstylae have been known to evolve into majority of the modern garden roses (Anonymous 1972). Eleven species of rose, *Rosa brunonii*, *R. eglanteria*, *R. foetida*, *R. gigantea*, *R.involucrata*, *R. leschenaultiana*, *R. longicuspis*, *R. microphylla*, *R. moschata*, *R. rubiginosa* and *R. sericea*are found in India in the wild (Nagar, 2007). This plant contains several plant secondary metabolites such as terpenes, glycosides, flavonoids and anthocyanins that have a lot of beneficial effects on human health. Mainly it has anti-oxidants and anti-inflammatory properties. Japan is known to be the largest cut-flower consuming country. Rose attar is known to be used widely in the perfumery and culinary industry. Rose is known to be used in the preparation of"Gulkand". Rose plant flowers three times a year (April-May, July-August and late October). The recalcitrant property of rose in the terms of morphogenesis from protoplast is known and only one report exists of whole plant regeneration from rose protoplast (Mattews *et al,* 1991).

The Hibiscus (*Rosa sinesis*) is known to be a national flower of Malaysia and a national symbol of Haiti and many other nations such as the Solomon Islands and Niue. The red hibiscus is the symbolic flower of the Hindu goddess Kali of Bengal, India. The hibiscus is used as an important offering to goddess Kali and Lord Ganesha in Hindu worship. Hibiscus is known to have anti-ageing and anti-acne properties. It also reduces hair fall, darkens hair and removes dandruff (Patel, 2010).

Traditionally, different rose cultivars are produced by sexually crossing the two species, also allowing them to have selected desirable traits in them but this method has a constraint and that is ploidy level difference. The major challenge in the conventional farming of Rose is, it is susceptible to frequent viral and fungal infections. *In vitro* regeneration is a promising approach to propagate disease free plants. In this context the present study has attempted to optimize the protocol for *in vitro* propagation of a rose species. The study has also extended to examine the protoplast fusion potential between the Hibiscus (*Rosa sinesis*) and Rose protoplasts towards the applications of developing somatic hybrids.

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MATERIALS AND METHODS

Plant Samples

Plant samples were collected from the garden at the Sir. M. Visvesvaraya Institute of Technology campus.

Processing of plant samples

The explants (nodes and internodes) were brought to the laboratory in sterile plastic bags. The nodes and internodes were excised from the plant using sterile scissors. After removing the leaves and buds from the explants with sterile forceps, it was washed under running tap water for 20 minutes. Then the explants were treated with 0.1% (v/v) Tween-20 for 15 minutes followed by a treatment with 0.5% (w/v) Bavistin for 45 minutes. It was then treated with 0.1% (w/v) of mercuric chloride for 5 minutes. At the end it was treated with 0.2% (w/v) PVP for 15 minutes followed by autoclaved distilled water wash for 3 to 4 times. After every surface sterilization step an autoclaved distilled water wash for 2 minutes, twice was performed.

Culture medium

For indirect organogenesis (shoot induction and proliferation of it) normal MS Media (Murashige and Skoog,1962) with 30g/l sucrose and 8g/l agar. Various concentrations of growth regulators 6-benzylaminopurine (BAP), Kinetin (Kn) were used in the MS medium (Murashige and Skoog 1962). The different combinations of hormones tested and the response is recorded in Table 1. Explants were inoculated on the semisolid autoclaved media (15psi, 121°C and 15minutes) and were incubated at a temperature of 25±2°C, under cool white fluorescent light with 16hrs photoperiod. Alternate weeks, 16 bottles of each hormone combinations were inoculated with 2 explants per bottle(to minimise competition of nutrition between explants in a single bottle). Cultures were monitored every day for any contaminations or response. Response was noted at the end of every 14-19 days. Contaminated samples were removed continuously and the responses of the remaining samples were considered.

For protoplast fusion of Rosa sinensis and Rosa sp.

The explants (leaves) of Hibiscus and Rose species were washed under running tap water for 5 minutes. Then the explants were treated with 0.1% (v/v) Tween-20 for 15 minutes followed by a treatment with 0.2% (w/v) Bavistin for 20 minutes. Next it was treated with 0.1% (w/v) of mercuric chloridefor 2 minutes. At the end it was treated with 0.2% (w/v) PVP for 15 minutes followed by autoclaved distilled water wash for 2 minutes, twice. After every step an autoclaved distilled water wash for 2 minutes, twice was performed. The lower epidermis of leaves was carefully peeled off. Then the leaves where then taken inside the laminar airflow and chopped into fine pieces.To the leaf samples protoplast isolation solution was added (0.5%) (w/v) macerozyme+2% (w/v) cellulase in 13% sorbitol or mannitol at pH 5.4) and was refrigerated for 1 day. The next day the solution was sieved out from the leaf sample due to the presence of a lot of mucilage. This solution was then taken into eppendorf tubes and microfuged for 4 minutes and the pellet was dispersed in autoclaved distilled water. For the protoplast fusion to be identified, to one of the samples (*Rosa sinensis*), dye methylene blue was added and incubated in room temperature for 10 minutes. 2ml of sample was taken from *each Rosa sinensis* and *Rosa sp.* and 2ml of PEG was added to it and was kept for incubation for 4 hours at room temperature.

RESULTS AND DISCUSSION

Different studies have shown different methods to surface sterilize plant samples. Surface sterilization protocol needs to be optimized for every different rose cultivar as different environment will harbor different microorganisms. For plant tissue culture the only challenge faced is to reproduce disease free, healthy plants for which the initial surface sterilization is important and also proper autoclaving of the media. Initially we followed a protocol devised by Saklani *et al* (2015) where they have used 0.2% (w/v) Bavistin for 20 minutes and 0.1% mercuric chloride for 2 minutes but we have observed a lot of contamination with this treatment. Few studies have suggested the treatment of explants with 70% (v/v) ethanol to reduce the contaminations (Khaskheli *et al* 2018; Sisko, 2011), but we have observed exposure to 70% (v/v) beyond 2 minutes was resulting into excessive drying of the explants, hence a very quick exposure to only 5-6 seconds rather just a simple wash with 70% (v/v) ethanol was followed for the samples. These manipulations in the surface sterilization protocols have reduced the contaminations drastically. PVP treatment was successful in reducing the browning of the medium due to polyphenol secretions.

The regeneration response to different hormone concentration is given in Table 2. The % response was calculated by total number of plants showing favorable response by total number of plants incubated.

We observed that with combination of BAP (3.5mg/l) and Kn (1.5mg/l) it showed better response than the hormones used alone (Table 2). Also it was noted that higher concentrations of both the hormones used alone or in combination tends to slower the response may be due to nonspecific action of the hormones and at times not give exuberant shoot regeneration. A study conducted by Saklani

Table 1: Response obtained from different hormone concentrations.

Growth hormones	Concentration of growth regulator	Response shown
MS+Kn	3.0mg/l	Slightly slower shoot regeneration mostly from the apical meristematic tissues
MS+Kn	1.0mg/l	Shoot regeneration mostly from apical meristematic tissues.
MS+Kn+BAP	1.5mg/l, 3.5mg/l	Multiple shoot regeneration, exuberant shooting.
MS+Kn+BAP	2.5mg/l, 5.0mg/l	Multiple shoot regeneration, slower response; not exuberant.
MS+BAP	5.0mg/l	Slow shoot regeneration.
MS+BAP	3.5mg/l	Shoot regeneration at a rate lower than combined hormones.

Fig 1: Fused protoplast of *Rosa sp.* and *Rosa sinensis.*

Table 2: Percentage of response to different growth regulators.

Hormones concentration	Total no.of samples inoculated	% Response obtained
Kinetin (3.0)	35	12.5
Kinetin (1.0)	35	25
Kinetin (1.5) and BAP(3.5)	35	62.5
Kinetin (2.5) and BAP(5.0)	35	37.5
BAP (3.5)	35	62.5
BAP (5.0)	35	37.5

et al (2015) reported the shoot regeneration of nodal explants in the combination concentration of BAP and Kn (5.0 mg/l+2.5 mg/l). Another study showed that almost 85% of shoot regeneration was observed on full strength MS medium containing (2 mg/l BAP + 1 mg/l Kn) and maximum average number of multiplied shoots was produced with a concentration of (3 mg/l BAP + 1 mg/l Kn) which was almost in consensus with our observations (Attia *et al*, 2012). Another observation made during this study was that, Kn was showing growth mostly from the apical meristems but with BAP was showing regeneration from nodes as well from internodes. Another report on *in vitro* regeneration has also advocated that the nodal segment explants are ideal for shoot regeneration with a hormone mixture of BAP an NAA (2.0mg/l+0.1mg/l) obtaining 100% shoot proliferation (Maurya *et al*, 2013). A similar study was conducted by Farahani *et al* (2012) recorded an excellent response using combination hormone of BAP and IBA (2.0mg/l+0.1mg/l). This can be inferred from our as well as the above mentioned studies that, combination of these hormones resulted in exuberant shoot regeneration was might be synergistically induced by both of these hormones also growth from both nodes/internodes, also apical meristemic tissues was seen from our method. Saklani *et al* (2015) used half strength M.S. media for rooting with a hormone mixture of NAA and BAP (2.0mg/l+0.5mg/l) showing a good effectiveness. Report from a study conducted by Attia *et al* (2012) showed that a hormone concentration of (2.0mg/l) IBA gave a 66% efficiency rate for rooting.

For the protoplast isolation and fusion, we obtained very good number of isolated protoplast from both the Rose species and *Rosa sinensis* took up the methylene blue dye successfully without affecting its own viability so we could successfully fuse them. Mesophyll tissue as a protoplast source eliminates the requirement for any callus culture or cell suspension culture steps before the isolation (Marchant *et al,* 1997).

The fusion took place on different planes (Fig 1) after the 4hours of incubation, though better fusion took place if we kept it for longer time (>4hours). A study by Pati *et al,* (2008) used a method of fusing protoplast using PEG and high concentration of calcium and reported seeing abnormal shapes of cell when compared with the control but we saw two completely different morphology of protoplast with or without fusion. They also stated that the time duration required for fusion was 15minutes maybe because of the addition of calcium but we failed to observe any such fusion within that time. *Rosa sinensis* being a round and *Rosa sp.* being a bit elongated cylindrical shaped. Since successful fusion was obtained and also when we tried to fuse a week old culture fusion was occurring so we can infer that methylene blue can be used for staining as it does not inhibit the viability of the cell. (Pati *et al,* 2008) also mentioned a method for heterokaryon selection and further characterization which could be carried out after the method we used since viability was not lost. This hybrid would have never been possible in nature since both of them have different ploidy levels.

CONCLUSION

From the study conducted on the optimization of micropropagation of the commercially important variety of rose, *Rosa sp.* it was inferred that with combined hormone concentration of BAP at 3.5mg/l and Kinetin 1.5mg/l obtained maximum response in terms of shoot proliferation from an explant on full strength MS media.

From the protoplast fusion carried out between *Rosa sp.* and *Rosa sinensis* it was found that using polyethylene glycol as fusogen and methylene blue dye to stain the protoplast of *Rosa sinensis* fusion was observed between the two species on different planes. With longer duration of incubation with PEG, more number of protoplast was seen to fuse.

The rose explants grown under optimum hormone concentrations can be hardened, acclimatized and transferred for field trails. *In vitro* propagation is an effective protocol for mass propagation of rose species that would greatly benefit the development of the Rose floriculture industry as there a is a high demand for rose oil and rose water based products for therapeutic and aromatic use. To overcome the issue of ploidy level in Rose hybrids, protoplast fusion is the best method to produce new and improved variety of roses. The positive result of a protoplast fusion between *Rosa sp*. and *Rosa sinensis* leads us to the possibility of new varieties of flowers of the rose family with improved characteristics in taste or aesthetic appeal and also may contain the therapeutic properties of both of them.

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Conflict of Interest

All the authors declare no conflict of interest with regards to content embodied in this paper.

REFERENCES

Attia, A.O., Dessoky, E.D.S. and El-Tarras, A.E., (2012). *In vitro* propagation of Rosa hybrida L. cv. Al-Taif Rose plant. African Journal of Biotechnology. 11(48): 10888-10893.

Balas, J., Coronado, P.A.G., Silva, J.A.T. and Jayatilleke, M.P., (2006). Supporting post-harvest performance of cut-flowers using fresh-flower-refreshments and other vase-water-additives. Floriculture, Ornamental and Plant Biotechnology*:* Advances and Topical Issues. 1: 612-629.

Boskabady M.H., Shafei M.N., Saberi Z. and Amini S., (2011). Pharmacological effects of Rosa damascena. Iranian Journal of Basic Medical Sciences. 14(4):295.

Broertjes, C. ed., (2012). Application of Mutation Breeding Methods in the Improvement of Vegetatively Propagated Crops (Vol. 2). Elsevier.

Fairbrother F, (1965)" Roses", Penguin, UK.

Farahani. F. and Shaker. S., (2012). Propagation and growth from cultured single node explants of rosa (*Rosa miniature*). African Journal of Plant Science. 6(10): 277-281.

Gudin S, (2000). Rosa: genetics and breeding. Plant Breeding Review. 17. 159-189.

Hurst CC, (1941). Notes on the origin and evolution of our garden roses. Journal of the Royal Horticulture Society. 66: 73-8.2.

Kajla, S., Kala, S., Kumar, A., Mir, H. and Singh, M.K.,(2018), Effect of growth regulators on *in vitro* shoot multiplication and plant regeneration of Rosa hybriad L. from Nodal Explants, International Journal of Current Microbiology and Applied Sciences.

Khaskheli A.J., Khaskheli M.I., Khaskheli M.A., Shar T., Ahmad, W., Lighari, U.A., *et al* (2018). Proliferation, multiplication and improvement of micro-propagation system for mass clonal production of rose through shoot tip culture. American Journal of Plant Sciences. 9(02): 296.

Krussman G, (1981). The Complete Book of Roses. *Timber Press, Portland, OR*

Marchant, R., Davey, M.R. and Power, J.B., (1997). Isolation and culture of mesophyll protoplasts from Rosa hybrida. Plant Cell, Tissue and Organ Culture. 50(2): 131-134.

Matthews, D., Mottley, J., Horan, I. and Roberts, A.V., (1991). A protoplast to plant system in roses. Plant Cell, Tissue and Organ Culture. 24(3): 173-180.

Maurya, R.P., Yadav, R.C., Godara, N.R. and Beniwal, V.S., (2013). *In Vitro* plant regeneration of rose (*Rosa hybrida* L.) cv„ Benjamin Paul through various explants. Journal of Experimental Biology. 1: 2S.

Mishra, N., Tandon, V.L. and Munjal, A., (2009). Evaluation of medicinal properties of *Hibiscus rosasinensis* in male Swiss Albino Mice. International Journal of Pharmaceutical and Clinical Research. 1(3): 106-111.

Murashige, T. and Skoog, F., (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plant. 15.

Nagar P.K., Sharma M., Ahuja .P.S, (2007). Rose: Some important findings with reference to physiology of flowering, Floriculture and Ornamental Biotechnology.

Patel, V.R., Patel, P.R. and Kajal, S.S., (2010). Antioxidant activity of some selected medicinal plants in western region of India. Advances in Biological Research. 4(1): 23-26.

Pati, P.K., Sharma, M. and Ahuja, P.S., (2008). Rose protoplast isolation and culture and heterokaryon selection by immobilization in extra thin alginate film. Protoplasma. 233(1-2): 165-171.

Saklani Kumud, Hem Pant, Vijay Rawat, (2015). Micropropagation of rose cultivars: Biotechnological application to Floriculture. Journal of Environmental Research and Development.10.

Shepherd, R.E., (1954). History of the Rose. (Vol. 77, No. 5, p. 416). LWW.

Šiško. M., (2011). Micropropagation of roses (Rosa spp.): the effects of different media on *in vitro* rooting. Agricultura (Slovenia). 8(2): 19-22.

Squirrell, J., Mandegaran, Z., Yokoya, K., Roberts, A.V. and Mottley, J., (2005). Cell lines and plants obtained after protoplast fusions of Rosa+Rosa, Rosa+Prunus and Rosa+Rubus. Euphytica. 146(3): 223-231.

Xia, Y., Deng, X., Zhou, P., Shima, K. and Teixeira da Silva, J.A., (2006). The World floriculture industry: dynamics of production and markets. Floriculture, Ornamental and Plant Biotechnology, Adv. Trop Issues. 4: 336-347.

Younis, A., Khan, M.A., Khan, A.A., Riaz, A. and Pervez, M.A., (2007). Effect of different extraction methods on yield and quality of essential oil from four Rosa species. Floriculture and Ornamental Biotechnology. 1(1): 73-76.