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. multiforma and R. wichuriana are 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. sericeae 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 (Matthews et al, 1991). The Hibiscus (Rosa sinensis) 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 sinensis) and Rose protoplasts towards the applications of developing somatic hybrids.

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% (w/v) Tween-20 for 15 minutes followed by a treatment with 0.5% (w/v) Bovine Serum Albumin (BSA) for 15 minutes. Then the explants were washed under running tap water for 5 minutes. Next the explants were treated with 0.1% (w/v) 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 inoculated 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% (w/v) Tween-20 for 15 minutes followed by a treatment with 0.2% (w/v) Bovine Serum Albumin (BSA) for 20 minutes. Next it was treated with 0.1% (w/v) of mercuric chloride for 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) Bovine Serum Albumin 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 (Khashkeli 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 the hormones used alone or in combination tends to slower the response may be due to

| 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. |
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)&BAP(3.5)  | 35                              | 62.5                |
| Kinetin(2.5)&BAP(5.0)  | 35                              | 37.5                |
| BAP(3.5)               | 35                              | 62.5                |
| BAP(5.0)               | 35                              | 37.5                |

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 4 hours of incubation, though better fusion took place if we kept it for longer time (>4 hours). 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 15 minutes 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 3.5 mg/l and Kinetin 1.5 mg/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 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 roses 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.

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