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An overview on the *in vitro* regeneration of *Canna*

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Abstract

Canna, the solitary genus of the family Cannaceae and order Zingiberales, comprises of about 51 species of flowering plants. It is a horticultural plant but its agricultural importance can't be undervalued. *Canna* is a self pollinating plant and require outside pollinator due to its typical flower structure. Propagation through seed germination is not considered reliable. In order to produce genetically modified and improved varieties of *Canna*, various *in vitro* techniques had been studied. Thus the present study reflects a comprehensive review on *in vitro* propagation of *Canna* species with special reference to some other members of Zingiberales.

Keywords*: Canna*; Cannaceae; Zingiberales; *in vitro* propagation; tissue culture

1. Introduction

Canna, the only genus of the family Cannaceae, is popularly known as an ornamental plant with beautiful flowers. Various morphological, cytological and taxonomical characteristics of family Cannaceae is closely related to other members of Zingiberales like Musaceae, Strelitziaceae, Lowiaceae, Heliconiaceae, Zingiberaceae, Costaceae and Marantaceae[1]. *Canna* is an important plant not only from the ornamental point of view but also it is an important plant for starch production as well as its medicinal values. From the primitive time, village people commonly use *Canna* as herbal medicines in their daily dealings. It is a perennial rhizomatus herb. Sexual method of propagation using seeds is not considered to be reliable because Cannas have a slow tendency for seed setting[2]. On the other hand if seeds were produced, they are either sterile or have extremely hard seed coat which contributes to their dormancy. This could be overcome by scarification of seeds before sowing. Scarification enhances germination but reduces the viability of seed[3]. Traditionally *Canna* is a vegetatively propagated root crop. The cultivation of this crop occurs through the division of rhizome. In *Canna*, vegetative propagation occurs through out the year but has some limitations. The conventional method of propagation is slow and susceptible to viral infection during multiplication[4-7]. These limitations might be overcome by modern propagation techniques. Micropropagation technique can be employed for rapid growth producing superior variety, which will be free from any contamination.

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2. In vitro propagation in *Canna*

2.1 Propagation using in vitro culture techniques

Plant tissue culture basically means the culture of plant tissue or cell in a sterile environment. *In vitro* cultured cell usually retains its potentiality to grow and establish into a whole plantlet. Regeneration of an organism from a single cell or a group of cells raises the importance of tissue culture. The basis of culture of vegetative cell having potentialities for the generation of an elementary organism clearly establishes the concept of totipotency. Thus, the phenomenon of totipotency indicates the techniques of cultivating isolated plant cells in nutrient solution to produce the whole organism. Later, it was studied that totipotency of a plant cell retained for a longer period, but still the property is not stable and usually lost some time after the isolation of the cells[8]. As a pioneer, Haberlandt[9], justified the totipotency of plant cell. Subsequently, artificial culture through meristemetic tissues[10], embryo culture[11] and successful rescue of embryos from seed culture[12] were performed in completely defined nutrient medium. This was followed by the establishment of phenomenon of precocious germination[13], thus, providing one of the earliest applications of *in vitro* culture.

With the development of techniques, two major events that revolutionized plant tissue culture were the

discovery of plant growth regulators like auxins and cytokinins and the formulation of nutrient media i.e. Murashige and Skoog or MS media[14]. MS media consists of macro and micro nutrients, carbon source, vitamins, salt and growth regulators. The MS salt formulation is now the most widely used nutrient medium in plant tissue culture. Again in 1974, Murashige[15] described the possible outcomes of micropropagation namely the formation of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Nevertheless, these findings set the stage for the dramatic increase in the use of *in vitro* cultures in the subsequent decades.

The culture of single cells by shaking callus culture in a conditioned medium gave rise to well established nurse cells[16,17]. Further research on single cell culture produced well defined cell colonies[18]. The above technique was widely used for cloning of cells, culture of protoplasts and induction of somatic embryos from the callus[19]. Later on *in vitro* culture of floral and seed parts was successfully established[20]. Protoplast isolation and fusion technology was developed for the first time during 1970s because of the commercially available cell wall degrading enzymes. This led to the isolation and fusion plant protoplasts. In 1985, plant protoplasts were isolated mechanically from the plasmolysed tissues[21]. The changes in the structure and physiology of cells in developing callus, cultured cells, and protoplasts had been carried out under light and electron microscope[22]. The fusion of isolated protoplasts was achieved in 1909. For the first time, Takebe and his coworkers revolutionized the whole world by exploring the totipotency of protoplasts. In tobacco, the fused protoplasts were regenerated and subsequently produce interspecific hybrid plant[23].

Gradually, *in vitro* methods were increasingly used as an addition to traditional breeding methods for the modification and improvement of plants. Production of variants is one of the important roles played by cell culture. In case of callus mediated organogenesis and somatic embryogenesis there is a possibility of producing variants and aberrant plants. Thus *in vitro* somatic embryogenesis, tends to be the most effective and rapid method of plant regeneration[24]. For the first time during 1970s, somaclonal variants have been utilized for plant improvement. Somaclonal variation in tissue culture is dependent on the variation in a population of cells either natural or induced in the artificial culture[25], or may be genetic or epigenetic and is usually observed in regenerated plantlets[26,27]. The variations in the regenerated plantlets have agricultural and horticultural significance and have been adopted for a number of economically and medicinally important plant species.

In the history of plant tissue culture, the growth and regeneration of isolated protoplasts to produce the whole angiospermic plantlets was initiated[28]. Subsequently, the fusion of protoplasts was standardized to generate superior plant through somatic hybrid formation. Protoplasts were fused mainly by two methods, one is physical method by using electric current i.e. electrofusion and other is chemical method by using polyethylene glycol i.e. PEG method. Both the methods were employed to produce somatic hybrid plants. But the major problem is the ability of hybrid cells to regenerate whole plantlet[29,30]. Protoplast fusion has been used to produce unique nuclear-cytoplasmic combinations which generally results in hybrid seed production, but till date the success is limited to a few species

2.2 Histological study

Somatic embryogenesis involves control of three consecutive steps:

- Induction of embryogenic lines from sporophytic cells
- Maintenance and multiplication of embryogenic lines
- Maturation of somatic embryos and conversion into viable plantlets[31].

Induction of embryogenic lines and their subsequent conversion into plantlets have been studied by many workers[32-34]. The steps involved in the multiplication of somatic embryo have been comparatively less studied although it directly contributes to the ability of the *in vitro* embryos for the germination and development of the complete plantlets[35]. Two major problems have been reported concerning the multiplication steps. The first one is the difficulty in obtaining stable and subculture-suitable lines that will produce embryos for a longer period of time[33,34]. The second problem is the lack of synchrony in embryo development and the risk of morphological abnormalities such as pluricotyledony, multiple apex formation and fused cotyledons etc.

Multiplication of embryogenic lines in angiospermic species can be achieved either by regular sub culturing of explants taken from compact or friable embryogenic calli[33], or by the formation of new embryos from the previously developed somatic embryos[31,34,36]. This second case is referred to as secondary embryogenesis.

In Quercus initiation of somatic embryogenesis has been described from a variety of sporophytic explants, namely stem segments, leaves and zygotic embryos. The multiplication of the embrogenic lines was first achieved from calli ageing on the same culture medium[37,38] or via successive transfers into fresh culture media with different growth regulator supplements[37,39]. Embryogenic response from anthers and ovary tissues was also obtained with similar procedures[40].

Researchers have noted that

- Within one embryogenic line the somatic embryos could occur from different histological origin, as observed for example in *Theobromo cacao*[41].
- The growth regulator composition of the culture medium influenced the histological origin of the somatic embryos in *Hevea brasiliensis*[42,43] and *Elaeis guineesis*[44].

Somatic embryos development and their histological studies were well discussed among the members of monocot[45,46]. Further, histology of *in vitro* somatic embryos was also studied in some close relatives of *Canna*. Novak and his coworkers described that the initiation of somatic embryo in banana occurred when basal leaf sheath and rhizome tissue were taken as explants for *in vitro* culture[47]. In most of the reports of embryogenesis, emphasis has been given to manipulate the nutrient composition, growth regulators in culture medium, physical conditions of incubation and other stress treatments to induce somatic embryos. Histology of some other Zingiberales like *Zingiber officinale*, Curcuma *mangga*, *Heliconia psittacorum* was also discussed[48-50]. However, till date no report was published on the development and histology of *in vitro* grown somatic embryos of *Canna*. The investigation of the histological origin and structural organization of the *in vitro* somatic embryos of *Canna* is yet to be done.

In plant tissue culture, the type of explant plays an important role in the regeneration process. In *Canna*, different plant parts were used as explant for *in vitro* culture. The most common explants used for *Canna* micropropagation were meristematic shoot tip, rhizome, terminal bud etc. The list of explants used for *in vitro* culture of some members of Zingiberales is given in table I.

In tissue culture, surface sterilization of the explants has a great importance as it is the first step to be taken to check exogenous contamination. The main objective of surface sterilization of the explant is to get rid of the fungal and bacterial contamination without hampering the biological activity of the explants. The commonly used disinfectants are ethanol, sodium hypochlorite, mercuric chloride etc. The type and concentration of the chemical to be used for sterilization and the incubation time of explant in the particular sterilant depends on the nature of explant[51]. The list of various disinfectants used in the tissue culture of *Canna* and some close Zingiberales is given in table I. Chemicals like extran, ethanol, sodium hypochlorite (NaClO), calcium hypochlorite [$Ca(ClO)_2$], mercuric chloride ($HgCl_2$), streptomycin sulphate [$(C_{21}H_{39}N_7O_{12})_2 \cdot 3H_2SO_4$] etc. were used in the *in vitro* culture of *Canna* and some close Zingiberales like banana, heliconia, turmeric and ginger (Table I).

The artificially prepared nutrient medium plays an important role in the successful growth and differentiation of excised plant tissues. The culture media is composed of several components like inorganic salts, macro and micro nutrients, vitamins, aminoacids, sugars, growth regulators (phytohormones), agar or gelrite. The minerals present in the plant tissue culture medium are used by the plant cell for the synthesis of organic molecules. The ions of different salts play an important role in transportation or osmotic regulation and in maintaining the electrochemical potential of the plant.

The requirement of nutrient varies not only among different plants but also for different parts of the same plants. Therefore, a multiple media may be required for optimal growth of all plant tissues. To overcome this, different nutrient solutions were proposed by different authors from time to time like MS medium[14], B5 medium[52], Banna micropropagation media (Readymade medium marketed by Hi-media) Nitsch medium[53], White's medium[54], Woody plant medium[55] etc. Consequently, the most suitable medium for optimal growth of a particular tissue could be determined by trial and error method.

Though a little work has been done in the area of *in vitro* culture of *Canna*, MS was considered as the best medium for the optimal growth and regeneration of Cannas. From the literature it was found that some authors used different strength and phases (e.g. solid, liquid phases) of MS medium. Nutrient media other than MS medium were also used. For the culture of terminal buds, Sakai and Imai used B5 medium, ½ MS and MS medium to establish a tissue culture system for *Canna edulis*[56]. Kromer and his co-worker used liquid MS, ½ MS and agar solidified MS media for rapid multiplication in *Canna indica*[57]. In case of Sakai and his co-worker the survival rate of the explant was highest in B5 medium where as in the *in vitro* study of Kromer and his co-worker liquid MS help in the optimal growth of explants. In both the above case solidified agar medium became least effective for the optimal regeneration of plants. A list of different explants and the regenerating medium for different Zingiberales is given in table I.

Sucrose is always supplied with the culture media as a source of sugar. Usually it is used in the form of carbon source at a concentration of 3% (w/v) in almost all tissue culture experiments. In various *in vitro* culture studies of *Canna*, sucrose was added at a concentration of 3% (w/v) except for the shoot tip culture of *Canna edulis* by Hosoki and Sasaki[4]. As a source of sugar they used 2% sucrose for the development of shoot and root. Similar report was also found in other Zingiberales like banana,

Table I:List of plant species with the sterilants, plant growth regulators, organic additives, incubation conditions, acclimatizing material used for its regeneration along with survival rate

Plant species	Explants	Effective culture medium	Sterilant used	Plant growth regulator	Additives	Temperature	Light	Potting mixture	Survival rate	References
Canna edulis	Shoot tip	MS	Detergent and 0.6% sodium hypochloride for 10 min	BA and IBA	No	28ºC	16 h illumination of 52 µmol·s^{-1}·m^{-2} of cool white fluorescent light	Porous sandy soil and 15N-8P-17K (2.5 g/l)	92%	Hosoki and Sasaki[4]
Canna edulis	Terminal bud	B5, ½ MS, MS	70% ethanol for 30s followed by 1% sodium hypochloride for 5 min	BA, NAA and TIBA*	No	28ºC	16/8h light/dark cycle with photosynthetic photon flux densities of 100 µmol m^{-2} s^{-1}	Sterile vermiculite:Commercial soil (1:1)	80-90%	Sakai and Imai[56]
Canna indica	Meristem	Liquid MS, ½ MS, MS agar solidified	-	Kinetin, Adenine sulphate, NAA and Ascorbic acid	Yes	6ºC (for 4 winter months)	-	-	-	Kromer and Kukulczanka[57]
Canna indica	Rhizome	MS	-	Kinetin and IAA	No	-	-	-	-	Kromer[61]
Musa spp.	Immature male flowers	MS	-	2,4-D, biotin and NAA	No	27ºC	16 h photoperiod (65 µmol m^{-2} s^{-1})	-	-	Assani *et al.*[59]
Musa paradisiaca	Shoot tip	Liquid MS	0.1% $HgCl_2$ for 5 min	BA and NAA	No	25ºC ± 2ºC	1000 Lux	-	100%	Ganapathi *et al.*[74]

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Plant species	Explants	Effective culture medium	Sterilant used	Plant growth regulator	Additives	Temperature	Light	Potting mixture	Survival rate	References
Musa paradisiaca (Cv. - Lacatan and Robusta)	Fruit tissue	White's media	-	2,4-D, 2,3,6-TPA*, BTOA*, Coumarin, Adenine sulphate and IAA	Yes (Coconut milk)	24.5ºC	-	-	-	Ram and Steward[58]
Musa paradisiaca (Cv.- Prata)	Lateral suckers	MS	-	BAP and IBA	No	-	-	Organic material, vermiculite and nutrient	Not known	Lameira *et al.*[62]
Heliconia bihai	Fruits	MS	70% (v/v) alcohol for 1 min followed by calcium hypochlorite solution	GA_3	No	25±2ºC	16 hour photoperiod for 52 days with a luminosity of 50 µmols $m^{-2}s^{-1}$	-	95%	Ulisses *et al.*[75]
Heliconia psittacorum	Shoot and bud	MS		2,4-D	No	26±2ºC	Dark condition	-	-	Goh *et al.*[76]
Curcuma longa	Sprout	MS	70% ethanol for 30s, 0.1% mercuric chloride for 15 min and 750 mg/l Streptomycin sulphate	Picloram, NAA, BA and TIBA, 2,4-D	No	25±2ºC	12.1 µm photons m^{-2} s^{-1} white fluorescent illuminated for 12 h	Sterile soil	90%	Salvi *et al.*[60]
Zingiber officinale	Young rhizome sprout	MS	0.2% (w/v) $HgCl_2$ for 10 min followed by 70% ethyl alcohol for 10 min	BAP, Kinetin and Zeatin	No	25±2ºC	16 h photoperiod with a light intensity of 2000-2500 lux in cool white fluorescent tubular lamps	Soil with potting mixture	94-100%	Bhattacharya and Sen[63]

NB*: TIBA- 2,3,5- triiodobenzoic acid, 2,3,6-TPA- 2,3,6- trichlorophenylacetic acid, BTOA – Benzothiazole-2-oxyacetic acid benzothiazole-2-oxyacetic acid

where 2% sucrose was suggested for the growth and regeneration of banana fruits[58].

In tissue culture, the quality of regenerated plantlets is dependent on the range of acidity or alkalinity of the culture media. The optimum pH for regeneration of plant varies with the type of explant used. Generally in various tissue culture experiments pH is maintained within 5.6-5.8. In case of *in vitro* culture of *Canna*, pH - 5.6 has been considered for the successful regeneration of explants[4, 56].

In plant tissue culture, there are three types of media are used namely solid, semisolid and liquid. A media becomes solid or semisolid depending upon the concentration of the solidifying agents used. Agar-agar which is obtained from algae like Gelladium or Gracilaria and gelrite, a naturally derived gelling polymer are most commonly used as soldifying agents. The media was solidified with agar 0.8% (w/v) in some of the cultures of *Canna*[4], where as in some other cases, lower concentration of agar i.e. 0.4% was used for better growth[57]. Gellan gum at a concentration of 2.5g/l was used as a gelling agent for *Canna edulis*[56]. Further, liquid media with filter paper bridge was also used in some of the *Canna* tissue culture experiments[56,57].

Plant hormones or plant growth regulators play a vital role in the optimum growth and regeneration of plants. Phytohormones are added to synthetic culture media in a very minute quantity and subsequently they tend to increase the level within the tissue. Usually, only a little amount of the synthetic hormones remain in the free form because most of the plant hormones are rapidly inactivated after uptake into the living tissue. It has been found that, in case of auxins, less than 1% of the hormone being present in the free form and rest exist in equilibrium between the free and conjugated form. The effect of hormones on the explant depends on the following factors

- rate of the uptake of hormone from the synthetic medium
- stability of hormone in the medium
- sensitivity of the explant tissue towards the hormone

The discovery and use of growth regulators like auxins, gibberlins, cytokinins and abscicins along with other organic additives created new dimensions in plant tissue culture. The role of growth regulators and their optimum concentration should be carefully chosen for obtaining desired responses in tissue culture. The major growth regulators used in plant tissue culture are auxins [indole-3-acetic acid (IAA), 1-napthaleneacetic acid (NAA), indole-3-buturic acid (IBA), 2, 4-dichlorophenoxyacetic acid (2,4-D), piloram etc], cytokinins [6-benzylaminopurine (BA), zeatin, kinetin, thidiazuron etc], gibberellins (GA_3, GA_4, GA_1, GA_7 etc), abscisic acid, ethylene etc. The list of plant growth regulators used in the tissue culture of *Canna* for the formation of callus, somatic embryo, shooting and rooting are provided in table I. The table also gives an idea about the effect of various phytohormones on the growth and regeneration of some of the Zingiberales like banana, heliconia, ginger and turmeric etc. The frequently used plant growth hormones in the regeneration of *Canna* are BA, IBA, NAA, KN, 2ip, IAA, 2,4-D etc. From the literature it was found that organic additives were not required for the growth of *Canna* except for Kromer and Kukulczanka[57], who used some organic supplements for the culture of *Canna indica* meristem. Further, coconut water is used as a supplement for the substantial growth in banana[58].

During aseptic culture of plant explant, various conditions for incubation play an important role for subsequent growth and development. In artificial culture, high temperature may lead to the disruption of the culture media and low temperature restricts the growth of tissue explant. Further, some tissue prefers to grow in light condition, while some other grows in dark. The intensity of light has a significant role in tissue regeneration. So an optimum temperature and light condition is required for the substantial growth of selected tissue explant. The incubation conditions followed by various researchers for regeneration of *in vitro Canna* and some other Zingiberales are shown in table I.

Callus is defined as an unorganized and undifferentiated mass of parenchyma cells formed from isolated plant cells or tissues under aseptic conditions. It is formed as a result of continuous division and growth of the explant tissue. Since meristematic cells have a capacity for vigorous growth and division, these tissues are used for the initiation of callus. So in plant tissue culture, young and immature parts of plant like leaf, stem, root, nodes and seeds are used for callus initiation. In the culture media the explant absorbs exogenously supplied nutrients and hormones and divides to form an unorganized mass of cells and tissues, which become enlarge and swell to rupture. This indicates the initiation of callus formation in the particular cells or tissues. As the cells rupture, the endogenous growth regulators along with the exogenously supplied hormone and nutrients stimulate further division of the cells. Thus the unorganized mass of callus tissue gradually increases its size.

In *Canna edulis*, embryogenic callus like structure was obtained by supplementing 1.5 - 2 mg/l BA in two different nutrient media (i.e. B5 and MS media). Those

callus like tissues were capable of growth and division, but failed to differentiate further and died soon thereafter. Where as, protocorm like structure was achieved by using 1 mg/l NAA in the same medium regenerating the complete plant by taking *in vitro* shoot tip as explant[56].

As literature of review did not find any reliable data regarding the initiation of callus in different species or cultivars of *Canna*, various protocol for induction of callus or similar structures in some close Zingiberales may be discussed. While studying two cultivars (Cv. Lacatan and Cv. Robusta) of *Musa paradisiaca*, Ram and his coworker reported the formation of fluffy calli from *in vitro* pulp of banana in white medium containing adenine sulphate and IAA. Unfortunately these calli didn't differentiate to produce organ[58].

A protocol for producing both friable and compact calli in *Musa* sp. from *in vitro* male flower was reported by Assani and his coworkers in 2002. They found white friable calli and yellow compact calli after 5-6 months culture of male flower in MS medium containing 4.1 µM biotin, 18 µM 2,4-D and 5.7 µM NAA. Both the calli were capable of vigorous division and finally regenerated into complete plantlet[59]. In case of turmeric, Salvi and his coworkers observed initiation of embryogenic callus from *in vitro* leaf base when cultured in MS medium supplemented with 2 mg/l picloram or 5 mg/l NAA in combination with 0.5 mg/l BA. The callus thus formed had potency for further growth, initiation of shoot and root and regeneration of whole plantlet[60].

In the later part of 1970s, Kromer studied that the addition of 2 ppm IAA and 1 ppm kinetin in MS medium was the best condition for the formation of shoot buds and regeneration of complete plant in *Canna indica*[61]. In the further study of *Canna indica*, Kromer and his coworker reported that supplementation of kinetin (2 mg/ dm^3), adenine sulphate (100 mg/dm^3) and NAA (0.2 mg/ dm^3) in MS medium was suitable for the initiation of auxiliary bud and subsequent formation of shoot and root to produce *in vitro* plant in a shorter period[57].

To promote the growth of shoot tip in *Canna edulis in vitro* culture, Hosoki & Sasaki (1991) observed that addition of 0.1 mg/l of BA in MS medium was optimum for shoot multiplication. Splitting of shoot after tip culture in the above mentioned concentration of BA increased the number of shoots. *In vitro* rooting initiated at a concentration of 0.1 mg/l IAA[4]. Another work on *in vitro* shoot tip culture of *Canna edulis*, Sakai & Imai reported that the survival rate of shoot was higher in a optimum concentration of IBA and BA (0.5 mg/l each) in B5 medium. Lateral shoot initiation was shown by supplementing BA and TIBA (0.5 mg/l each) to the culture medium. The rate of rooting was highest, when the media was supplemented with NAA (0.1 mg/l)[56].

The initiation of shooting and rooting was studied by various authors among different members of Zingiberales. Lameira and his coworkers in 1990 carried out *in vitro* culture of *Musa paradisiaca* (Cultivar- Prata) taking lateral suckers as explants. They noticed shoots were obtained with 2.5 mg/l BAP where as rooting was initiated by supplementing 5mg/l IBA in MS medium[62].

Salvi and his coworkers reported that shoot multiplication in *Curcuma longa* was started after 2 months of inoculation of sprouts with BA (1 mg/l) and NAA (0.1 mg/l). Auxiliary buds were initiated from the cultured shoot bud. Further elongation of shoot bud and development of root was observed from the excised shoot bud in MS medium without any phytohormone. *In vitro* generated callus was shown to produce shoot primordia by supplementing BA (5 mg/l) in combination with TIBA (0.1 mg/l) or 2,4-D (0.1 mg/l). Callus produced by the combination of BA (0.5 mg/l) and NAA (5 mg/l) showed highest percentage of response for shoot primordia when those excised callus were cultured in a medium containing BA and TIBA (90%) than that of BA (≤5%) alone or in combination with 2,4-D (75%). Thus TIBA, an antiauxin was proved to be beneficial for regeneration of *in vitro* turmeric plant. Further development of shoot was reported when subcultured in ½ MS with 2% sucrose in combination with kinetin (1 mg/l)[60]. In case of *Zingiber officinale,* Bhattacharya and Sen (2006) used *in vitro* rhizome as explant and achieved maximum rate of shooting and rooting by the addition of BAP (4 mg/l) in MS medium than that of B5 medium. Maximum number of plantlets and their maximum height was observed in presence of BA, where as kinetin and zeatin showed moderate effect on number and height of plantlets[63].

Somatic hybridization seems to be a promising complement to classical breeding since protoplasts are amenable to complete plant regeneration. Further, protoplast fusion parameter for establishment of somatic fusion technology is one of the strategies for genetic improvements in plant tissue culture. Literature of review did not give any document on protoplast isolation and their fusion technology among different species of *Canna*. Till date, somatic hybrid formation and fusion process is also not so common among other members of Zingiberales except in banana. Protoplasts were isolated from young leaves, friable calli and cell suspensions of different diploid and triploid cultivars of *Canna*. Protoplasts isolated from cell suspension culture developed into complete plants where as mesophyll protoplasts and callus derived protoplasts were incapable of regeneration. The yield ($27.5*10^6$ protoplasts per ml of cell volume) and the viability of cell suspension

protoplast (71-91%) were much higher than that of mesophyll and calli protoplasts (yield approx. $2.8*10^6$ and viability approx. 27-40%). Somatic embryo was produced after 8-10 weeks of protoplast culture and plants were observed after 11-12 weeks after the subculture of somatic embryo[59]. However, it was Papadakis and his coworkers who found that isolated plant protoplasts in tobacco and grapevine had lower regenerating capacity because of suppression of totipotency. Reduced viability and cell division potential in isolated protoplasts was because of the presence of higher contents of reactive oxygen species and oxidized form of some of the antioxidant enzymes like ascorbate and glutathione etc. They proved that the reduced cellular antioxidant mechanism was significantly correlated with the suppression of expression of isolated protoplasts[64]. Yasuda and his coworkers observed similar result while isolating *Brassica napus* leaf protoplasts. The increase in the intracellular reactive oxygen species during the isolation of protoplasts will result in the apoptosis like cell death of the cultured protoplasts[65].

Further study of protoplast fusion in banana, Assani and his coworkers in 2005 studied the fusion of banana protoplasts in two different methods, namely electrofusion method (by alternating current field) and chemical method (by poly ethylene glycol). The fusion efficiency was found to be higher in chemical procedure (17%) than that of electric method (10%). Optimum concentration of poly ethylene glycol (PEG) for banana protoplast fusion was also studied. According to them, more than 50% of PEG leads to severe damage of the protoplsts and finally hamper the process of fusion of cells. Further, its application longer than 30 min also hampers the fusion of protoplsts. Again it was observed that the cell division rate was higher in electric method (35%) than that of chemical method (24%). Some other aspects of banana protoplast fusion like rate and duration of somatic embryogenesis and plantlet regeneration were also higher in electric fusion technology[66].

Tissue culture is extensively employed in the production, conservation and improvement of plant resources. In tissue culture, somaclonal variation is known to be an inherent variation in a population of cells usually observed in regenerated plants[25]. Somaclones produced in plant tissue culture seems to have two contrasting aspects. On one hand, it affects the use of tissue culture negatively by hampering the conservation of plant resources and on the other hand it is a source of new desirable clones or variants with better agronomic traits. To date somaclonal variation in *Canna* is not reported. However this process was well studied among the close Zingiberales like banana, turmeric and ginger. Various RAPD primers were used to detect somaclonal variation by comparing the DNA profiling of parent plant with *in vitro* regenerated plantlets. Increase in the time period of the sub-culturing increase the possibility of occurrence of more genetic variations. The occurrence of specific bands or loci in the regenerated plants of different sub-cultures may be used in the genetic identification of the somaclones[67,68]. Somaclonal variation among *in vitro* regenerated plants of turmeric was also reported[60]. In contrast, genetic uniformity among *in vitro* regenerated cultivars of banana was observed using various ISSR markers[69]. Genetic fidelity was also observed among other Zingiberales like ginger and turmeric[70,71].

The successful transfer of *in vitro* generated plants from laboratory to field condition is an important part of the entire tissue culture experiment[72]. Drastic environmental changes occurred when the *in vitro* plants are transferred to open field condition. *In vitro* condition provides low light intensity, high humidity and poor root growth, where as under field or green house condition there is higher light intensity, low humidity with various soil microflora[73]. Several protocols have been suggested by different tissue culturists to overcome some of these obstacles.

In hardening of *in vitro* regenerated plantlets of the *Canna* and some related Zingiberales, different hardening materials like porous sand, sterilized potting soil, vermiculite, commercial soil, organic material, nutrient and manure (NPK) etc. have been used (Table I). The success rate of hardening depends upon the hardening material and the condition of the regenerated plantlets. High rate of survival of regenerated plantlets have been achieved in field condition.

Conclusion

A series of work have been carried out by different scientists on various aspects of *in vitro* propagation of *Canna* and some other members of Zingiberales. Traditional breeding has been greatly hampered by the contamination of virus and insects. Literature of review does not provide the exact techniques of *in vitro* propagation of *Canna*, still the published information of *Canna* and other Zingiberales gives an idea about various important parameters of micropropagation. Thus, through micropropagation new plants can be regenerated in a sterile environment within a short period of time which will be free from any virus or insect infestation. Further research will result in genetic improvement in *Canna* by establishing new variety, development of efficient methods for mass production of superior quality planting stock and conservation of the genetic resources.

Conflict of interest

The author's declares none.

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