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RESEARCH ARTICLE

IN VITRO PLANT REGENERATION FROM THE CALLUS CULTURES DERIVED FROM HYPOCOTYL SEGMENTS OF BRASSICA OLERACEAE L VAR BOTRYTIS CV EARLY KUNWARI

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ABSTRACT

The development of *in vitro* propagation of plants holds tremendous potential for genetic manipulation of crops and other applications. In the present study, attempts have been made to develop a simple, reliable and reproducible protocol for micropropagation from different explants of *Brassica oleraceae* L *var botrytis*. Callusing at a frequency of 100% was observed when hypocotyls segments derived from 6-days-old seedlings of cauliflower were cultured on MS medium supplemented with 2,4-D (1mg/L) and Kinetin (1mg/L). Three months old calli were regenerated at a frequency of 25% when subcultured on MS medium supplemented with just kinetin (1mg/L) alone. Regenerated plants grew well when transferred to soil.

INTRODUCTION

Tissue culture involves the use of small pieces of plant tissue (explants) which are cultured in a nutrient medium under sterile conditions. Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce new shoots, and, with the addition of suitable hormones new roots. These plantlets can also be divided, usually at the shoot stage, to produce large numbers of new plantlets. The new plants can then be placed in soil and grown in the normal manner. Micropropagation of various plant species, including many medicinal plants has been reported (Haccius, 1978; Mukhopadhyay *et al.,* 1991; Eimert and Seigmund, 1992; Kumar *et al.,* 1993; Fransza *et al* 1994). Plant regeneration via somatic embryogenesis from single cells, that can be induced to produce an embryo and then a complete plant, has been demonstrated in many plant species. Somatic embryos, which are bipolar structures, arise from individual cells and have no vascular connection with the maternal tissue of the explants (Haccius, 1978). Embryos may develop directly from somatic cells (direct embryogenesis) or development of recognizable embryogenic structures is preceded by numerous, organized, non-embryogenic mitotic cycles (indirect embryogenesis). Somatic embryogenesis has a great potential for clonal multiplication. Under controlled environmental conditions, somatic embryos germinate readily, similar to their zygotic counterpart. Mukhopadhyay *et al.* (1991) developed a reliable protocol for plant regeneration from the protoplasts derived from hypocotyl segments of *Brassica oleraceae*. Healthy viable protoplasts could be isolated from hypocotyl tissues of 6-day-old seedlings. Any variation in the age of the seedlings drastically reduced the yield. Calli, when transferred to rooting medium, differentiated multiple shoots in about 80% of the cultures in 3-4 weeks. These shoots were rooted with 100% efficiency within 7 days of transfer to rooting medium. They also achieved 10 - 33 % genetic transformation subjecting protoplasts to PEG mediated direct uptake of plasmid DNA. Cauliflower is one among the members of the very useful genus *Brassica*. It is grown worldwide as one of the vegetable crops. So any improvement in this crop will definitely benefit human population to a considerable extent. Major objective of this study is to develop a reliable and reproducible protocol for callus initiation and *in vitro* plant regeneration from the hypocotyl segments.

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MATERIALS AND METHODS

Seeds of cauliflower (*Brassica oleraceae* L *var botrytis* cv Early Kunwari) were obtained from Ottanchatram, Dindigul district, Tamilnadu, India. Seedlings used for tissue culture experiments were grown aseptically on MS basal medium (solidified) (Murashige and Skoog 1962). Seeds were disinfected for 5 minutes with 70% ethanol and 0.1% $HgCl_2$ for 15 minutes and washed several times with sterile deionised water before germination. Seeds were then incubated under dark at 25°C for 3 days and transferred to a photoperiod of 14 hour light and 10 hr dark. Hypocotyl segments (0.5 – 1.0 cm long) of thus grown seedlings were used for callus induction. Cultures were kept under controlled physical environment at 25°C and a photoperiod of 14 hour light and 10 hour dark. Culture racks were illuminated with

cool fluorescent tubes. Calli were subcultured after every four weeks on the same medium. Three-month-old callus cultures were used to study regeneration ability. MS medium supplemented with various concentrations of growth hormones were made and the calli placed over them. Regenerative ability of calli under different combinations of growth hormones was studied after 40 days of incubation.

RESULTS AND DISCUSSION

The successful determination of the role of plant growth hormones, particularly, the ratio of auxins and cytokinins in the growth medium in directing cell differentiation by Skoog and Miller (1957) many successful efforts were made to develop methodologies to dedifferentiate somatic plant tissue to a mass of undifferentiated cells called callus and to redifferentiate these callus cells into complete plants, a property termed as totipotency, which is exhibited by all somatic cells of higher plants. Though, theoretically each and every somatic cell is totipotent, it has been observed/reported in various studies that, totipotency of the cells is restricted by various factors like genotype of the cultivars and *in vitro* culture conditions like nutrients, hormone compositions, growth conditions, source and age of explants. These conditions need to be optimized in order to develop a reliable protocol in order to obtain *in vitro* plant regeneration from cells/tissues for both monocot and dicot species including cauliflower.

Different explants like hypocotyls (Mukopathyay *et al* 1991) and petiole (Metz *et al.,* 1995a, b) have been successful in inducing embryogenic callus in cauliflower. Further MS basal medium supplemented with 2,4-D alone (0.1 mg/L) (Eimert and Seigmund, 1992) or in combination with Kin (1 mg/L) (Mukopathyay *et al* 1991) and BAP (2 mg/L) alone (Metz *et al.,* 1995a, b) have been used successfully for embryogenic callus cultures from different explants. In this study, out of various combinations tested 2,4-D (1mg/L) along with Kin (1mg/L) in MS basal medium gave 100% callus in hypocotyls explants.

To find out the optimum concentration of 2,4-D and Kin to induce callusing from hypocotyl explants (0.5 – 1.0 cm) were allowed cultured on MS basal medium supplemented with different concentrations of 2,4-D and kinetin. Out of the combinations tested 1 mg/L 2,4-D and 1 mg/L Kin proved to be the best for the induction of callusing as it shows 100% callusing (Table 1). Further, an attempt was made to study the effect of age of explants on callusing response. Hypocotyls from 6, 8 and 10 days old seedlings were cultured on MS basal medium supplemented with 2,4-D (1mg/L) and Kin (1mg/L). It was observed that induction of callus was age dependent which decreased with increasing age of explants (Table 2). It has already been demonstrated that different explants derived from the same plants differ in callusing response.

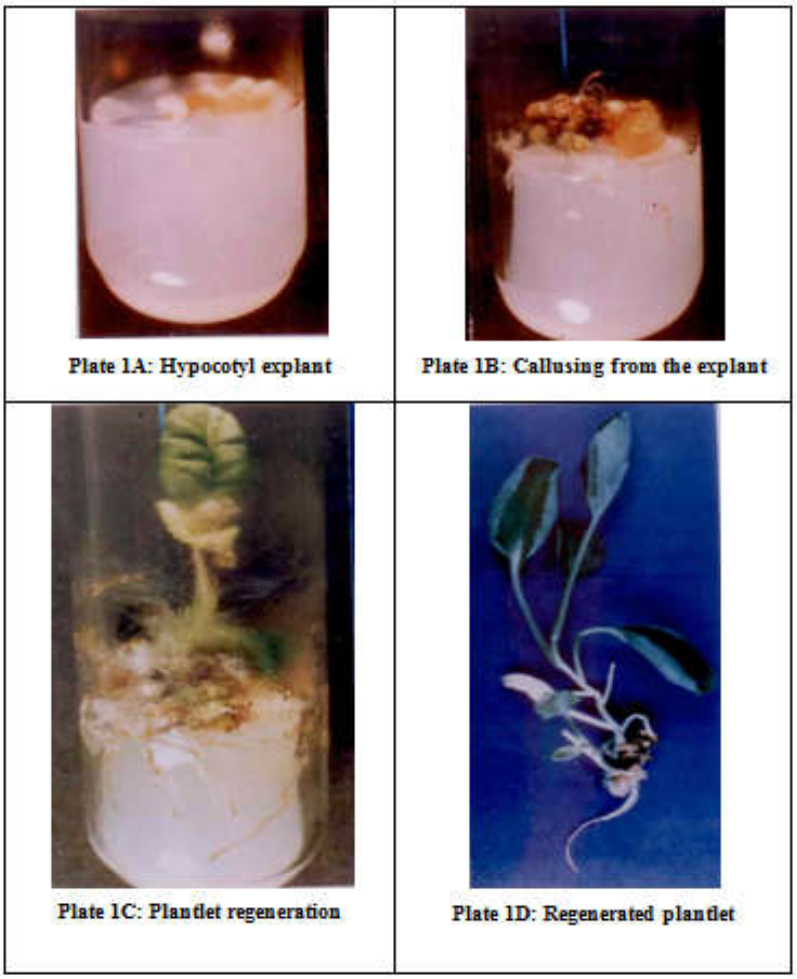

Plate 1: Callus induction and plantlet regeneration the from the hypocotyl segments of 6-day-old seedlings of cauliflower.

Table 1: Induction of callus from hypocotyl explants

Hypocotyl explants derived from 6-day-old dark grown seedlings were cultured on MS basal medium supplemented with different concentrations of 2,4-D and Kinetin. Observations were made after 30 days.

Hormone combination (mg/L)	Number of explants inoculated	Number of explants showing callusing	Frequency of callusing (%)
1 mg/L 2,4-D 1 mg/L Kin	60	60	100
1 mg/L 2,4-D 2 mg/L Kin	60	45	75
1 mg/L 2,4-D 3 mg/L Kin	60	21	35
1 mg/L 2,4-D 4 mg/L Kin	60	-	-
1 mg/L 2,4-D 5 mg/L Kin	60	-	-
2 mg/L 2,4-D 1 mg/L Kin	60	39	65
3 mg/L 2,4-D 1 mg/L Kin	60	18	30
4 mg/L 2,4-D 1 mg/L Kin	60	-	-
5 mg/L 2,4-D 1 mg/L Kin	60	-	-

Table 2: Effect of age on the hypocotyl explants in the callusing

Hypocotyl explants derived from 6, 8 and 10 day-old seedlings were cultured on MS basal medium with 2,4-D (1mg/L) and Kin (1 mg/L). Observations were made after 30 days. Experiment was done with 60 replicates

Age of the explants	Frequency of callusing (%)
6 days	100
8 days	81.7
10 days	53.7

Table 3: Effect of different explants on the induction of callus

Different explants like hypocotyls, epicotyls and first leaf segments were cultured on MS basal with 2,4-D (1mg/L) and Kin (1 mg/L). Observations were made after 30 days; experiment was done with 60 replicates.

Explants	Frequency of callusing (%)
Hypocotyls	100
Epicotyls	45
First leaf	NR

NR = No response

Table 4: *In vitro* plantlet regeneration from callus derived from hypocotyl

Ninety days old rapidly proliferating callus derived from 6-day-old hypocotyls was transferred to MS basal medium supplemented with different combinations of auxin and cytokinin. Observations were made after 40 days

Hormone combination (mg/L)	Number of callus clumps	Number of calli showing regeneration response	Frequency of regeneration (%)
1 mg/L NAA 1mg/L BAP	80	-	-
1 mg/L NAA 2 mg/L BAP	80	-	-
1 mg/L NAA 4 mg/L BAP	80	-	-
2 mg/L NAA 1 mg/L BAP	80	-	-
4 mg/L NAA 1 mg/L BAP	80	-	-
1 mg/L IAA 1mg/L BAP	80	-	-
1mg/L Kin only	120	30	25

To know the best explants for the induction of callus, hypocotyl segments, epicotyl segments and the first leaf bits from 6-day-old seedlings were cultured on MS basal medium with 2,4-D (1 mg/L) and Kin (1 mg/L). It was observed that hypocotyl segments showed 100% callusing whereas epicotyls showed only 45% callusing response. The first leaf segments did not show any callusing response (Table 3). Fransza *et al* (1994) noticed that the regeneration rate is strongly affected by the hormonal composition of the callus proliferation medium. Plant regeneration has been obtained

by transferring to MS basal medium supplemented with 0.4 mg/l NAA and 1 mg/L BAP (Eimert and Sigmund, 1992). Metz *et al* (1995a, b) observed shooting response on the same medium as that of callus induction medium within 4-6 weeks, which developed rooting when transferred to medium without BAP, where as Mukopathyay *et al.,* (1991) observed shooting, when callus was transferred to MS medium with 1 mg/L kin (without 2,4-D) and rooting was induced when shoots were transferred to hormone free medium. A rapid single step method for complete plantlet regeneration in cauliflower is described. Curd explants cultured on Murashige and Skoog (MS) medium supplemented with 1 mg-1 indoleacetic acid (IAA) developed complete plantlets in 25 days. Regeneration potential of curd explants was drastically reduced with increasing storage of cauliflower curds at room temperature beyond 8 days of harvesting (Kumar *et al.,* 1993). However, in this study, we observed 25% plant regeneration in the callus cultures when they were transferred to MS medium with Kin (1mg/l) alone within 6 weeks.

Various combinations of growth hormones were tested for *in vitro* plant regeneration in callus derived from hypocotyl explants. Callus cultures were maintained for 90 days (two subcultures of 30 days each on callus induction medium) before transferring to plant regeneration medium which had MS basal medium with different combinations of auxin and cytokinin. It was observed that the mere withdrawal of 2,4-D from the callusing medium could induce plant regeneration response at a frequency of 25% (Table 4, Plate 1). The regenerated plantlets were established after hardening. Further, effect of age of callus on plant regeneration was also studied. When the callus cultures derived from hypocotyls, were transferred to plant regeneration medium (MS + Kin 1 mg/L) after 3, 4, 5, 6, 8 and 10 months plant regeneration could be routinely observed up to 6 months at 25% frequency. After this, ability of plant regeneration decreased as only rooting is observed in 8 and 10 months old calli (Table 5). The period of callus proliferation is therefore considered as a critical step in shoot differentiation (Fransza *et al.,* 1994).

Conclusion

In conclusion, a simple, reliable and reproducible protocol for callus induction from the hypocotyls segments of 6-day-old seedlings of cauliflower has been developed. Callusing at a frequency of 100% was achieved. Three months old calli were regenerated at a frequency of 25% when they were transferred to a medium with just 1 mg/L kin. The regenerated plantlets were established after hardening.

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REFERENCES

Eimert K., and Siegemund F. 1992. Transformation of cauliflower (*Brassica oleracea* L. var. botrytis) - an experimental survey. Plant Mol Biol. 19 (3): 485-90.

Fransza, P.F., Leunissena, E.H.M., Van Eeuwijkb, F.A., Keizerb, P.L.C., and Colijn-Hooymans, T.M. 1994. Improvement of protoplast regeneration from a recalcitrant inbred line of Brassica oleracea: a morphogenic analysis. Plant Science 98 (1): 87–95

Haccius, B. 1978. Question of unicellular origin of non-zygotic embryos in callus cultures. hytomorphology 28 (1) 74-81.

Kumar, A., Kumar, Kumar, V.A., and Kumar, J (1993). Rapid *in vitro* propagation of cauliflower. Plant Science 90 (2): 175–17

Metz, T. D.; Dixit, R.; Earle, E. D. 1995a. *Agrobacterium tumefaciens* - mediated transformation of broccoli (*Brassica oleracea var italica*) and cabbage (*B. oleracea var capitata*). Plant Cell Rep. 15:287–292.

Metz, T. D.; Roush, R. T.; Tang, J. D.; Shelton, A. M.; Earle, E. D. 1995b. Transgenic broccoli expressing a *Bacillus thuringiensis* insecticidal crystal protein – implications for pest resistance management strategies. Mol. Breed. 1:309–317.

Mukhopadhyay, A, R Topfer, A K Pradhan, Y S Sodhi, H H Steinbiss, J Schell and D Pental. 1991. Efficient regeneration of *Brassica oleracea* hypocotyl protoplasts and high frequency genetic transformation by direct DNA uptake. Plant Cell Reports 10: 375-379.

Murashige T and Skoog F. 1962 A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15: 473-479.

Skoog, F., and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp. Soc. Exp. Biol. 11, 118–131.

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