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Differentiation of Multiple Shoots and Roots from Anther-Derived Callus Masses in *Antirrhinum majus* L.

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ABSTRACT

Anthers containing uninucleate pollen grains were cultured on MS and NB basal media supplemented with different auxins (IAA, NAA, 2,4-D) and cytokinins (BA and Kn) in various concentrations and combinations. MS medium was superior to NB medium in enhancing frequency of callus formation in anthers. Multiple shoots and roots were produced during subculture of callus on fresh medium supplemented with BA.

Keywords: Multiple Shoots, Anther, Callus, MS and NB Media

Antirrhinum majus L. is commonly known as Snapdragon or Dog Flower that belongs to the Family Scrophulariaceae. It is commonly grown in gardens as ornamental plants in winter. Lots of work is being done on gene expression, transposons as well as sequencing of the genome. Sofar little work has been done on *in vitro* propagation of *Antirrhinum majus* (Sangawan and Harada, 1975, Newbury 1986; Atkinson *et al*. 1989 and 1991; Okubo *et al.* 1991; Sheyab, *et al.* 2010). No study has been reported on the anther culture in this plant. However, anther culture studies have been done in several other plants belonging to other families like Solanaceae and Poaceae with the objective to produce haploid plants. In a recent paper, regeneration of plants has been reported from callus derived cotyledon cultures of *Antirrhinum majus* (Hesami and Daneshvar, 2016). Barinova *et al.* (2002) have developed protocol for manipulating isolated unicellular microspores of *Antirrhinum majus*, which include *in vitro* maturation, biolistic transformation and transient assay of gene expression, germination of pollen *in vitro* and *in situ*, cytological tests such as DAPI, FDA and aniline blue stains.

MATERIALS AND METHODS

Seeds were initially obtained from Government Sunder Nursery, New Delhi, and were grown in our botanical garden from November to December. Under climatic conditions of Delhi, *Antirrhinum* flowers between February and April. Floral buds of varying lengths were collected from the garden grown plants and then washed thoroughly with tap water. A correlation between the floral bud length and stage of microspore/ pollen was derived by squashing anthers from buds of different lengths using 2% acetocarmine. For sterilization flower buds were treated with saturated chlorine water for 15-20 minutes and then washed 3-4 times with sterilised distilled water. The anthers were then dissected from buds and placed on the surface of a semisolid medium. All steps from sterilization up to inoculation were carried out under aseptic conditions in a laminar flow cabinet.

MS (Murashige and Skoog, 1962) and NB media (Nitsch, 1969) have been used. They contained 3% sucrose (BDH, London) and 0.8% agar (Difco-Bacto, USA). Different auxins such as IAA, NAA and 2,4-D and cytokinins like BA and Kn were added to media either alone or in different combinations. The pH of media was adjusted to 5.8 before autoclaving. The sterilization of media was done at 15 lb/inch^2 for 15 minutes at 121 degree celsius temperature. All cultures were maintained under continuous cool white light (approximate intensity 1000-1500 lux) emitted by two or three fluorescent tubes(Phillips, 40 watts). The culture room is maintained at 24-27^0 C. The relative humidity of the culture room was maintained between 45 and 55%.

Callusing anthers were observed at different intervals. Data was collected in terms of (i) Numbers of anthers inoculated, (ii) Numbers of cultures survived, (iii) Number of anthers callusing and their percentage response, (iv) Number of cultures producing root/ shoot and their percentage of response, (v) Length and thickness of root/shoot, (vi) Amount of callus by measuring callus size with the help of a graph paper, and (vii) presence or absence of callus nodules.

OBSERVATIONS

Morphogenic response on MS medium

MS medium was superior to NB medium in enhancing frequency of callusing anthers as well as the amount of callus per anther. Within 10 to 25 days of inoculation on hormone supplemented media,most of the anthers became light green or yellowish green and swell. After a month of cultures, responsive anthers started callusing. The nonresponsive anthers did not even change their size and colour. On MS basal medium only a few anther produced callus. However, when supplemented with BA and 2,4-D either individually or in combination, the frequency of callusing anthers increased significantly. The highest percentage of callusing anthers was 85% on MS + 0.22 mg/l + 1 mg/ml 2,4-D. However, they did not undergo any organogenesis on MS basal or even hormone supplemented media. The callus initiated from different regions of stamen such as connective, anther locules and the filaments.

Morphogenic response on NB medium

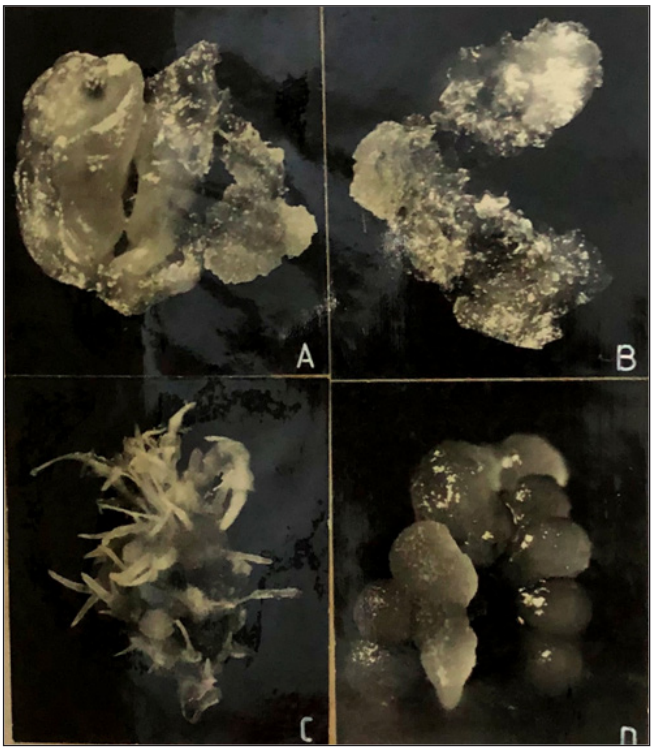

Fig. 1: Morphogenetic response of anthers cultured on NB medium supplemented with 1mg/l 2,4-D. **(A,B)** Callus developed from locule surface and filament after 90 days of inoculation. A × 12, B × 16.6 **(C)** Differentiation of roots and white hair after 125 days of inoculation. × 3.8 **(D)** Globular nodules produced directly on anther after 84 days of inoculation × 19

Anthers were cultured on NB medium supplemented with 2,4-D (1 and 2 mg/l) and cytokinins (BA and Kn) either alone or in various combinations. Callusing was observed in all the media tried. With 8-10 days of culturing, anthers became swollen and started callusing and gradually formed callus masses of various sizes (Fig.1A,B). In some of the cultures, globular nodules were produced (Fig.1D). Initially calli were green or yellowish green but later after 113 days, turned brown. Prominent roots and numerous white hairs developed on calli within 37 days on NB, NB + BA (0.22 and 2.25 mg/l), NB + Kn (0.21 and 2.15

mg/l) and NB + Kn (0.21 and 2.15 mg/l) + 2,4-D (1 and 2 mg/L). The maximum number of roots per culture (25) developed on NB + 2.15 mg/l Kn + 1 mg/l 2,4-D (Fig.1C).

Subcultures on MS medium

Callus masses obtained on MS media were subcultured for several passages on MS basal as well as BA, Kn, and IAA supplemented media. In each subculture, calli increased in size and number of roots per culture also increased to 50 and maximum length approached 4 cm. Some of the calli also differentiated shoots on MS+1mg/l BA medium.

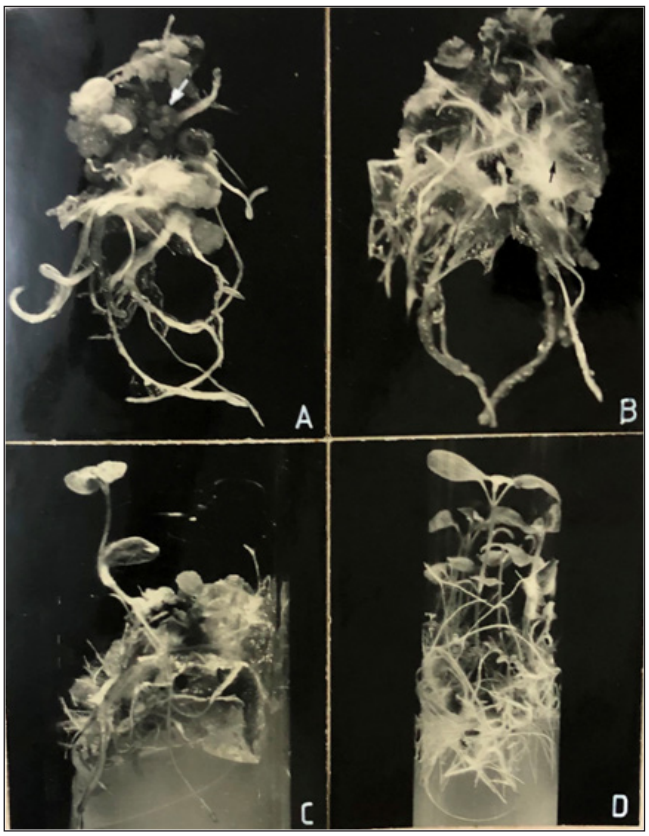

Fig. 2: Organogenic responses of subcultured calli of *Antirhhinum majus* anthers on NB medium. **(A)** Callus nodule and roots developed after first subculture of anther-derived callus in 25 days, × 3.2. **(B)** Roots and white hairs in callus subcultured after 33 days, × 3.3. **(C)** Differentiation of young shoot and roots after 150 days of anther inoculation, × 2.5. **(D)** Multiple shoots and roots formation after third subculture on NB + 0.1 mg/ l BA after 268 days of anther inoculation

Subcultures on NB medium

Callus obtained on NB medium containing various concentrations of 2,4-D (1-8 mg/l) was subcultured on NB basal medium where shoots were differentiated along with nodular callus masses, roots and numerous white hairs. Shoots were differentiated within 3-4 weeks from initial subculturing (Fig.2 A,B). The number of shoots per culture was 1or 2 and the frequency of cultures producing shoots was 4% (Fig.2C). The second subculturing on NB + BA(0.1 and 1mg/l) and NB + 1mg/l BA=0.1 mg/l NAA produced shoots at higher frequency (9%). In another set of experiments, initial callus developed on NB + 1mg/l 2,4-D when subcultured on NB + 0.1mg/l BA formed multiple shoots during third subculture (Fig.2D). Thus multiplication of callus derived plants has been achieved (present study) by repeated subculturing on NB medium and a protocol has been developed for obtaining plants from anther cultures of *Antirrhinum majus L.*

RESULTS AND DISCUSSION

Both MS and NB media are good for differentiating callus masses from cultured anthers in *Antirrhinum.* 2,4-D alone or in combination with BA and Kn is effective for callusing. MS medium was better than NB medium in enhancing frequency of callusing anthers as well as amount of callus per anther. Successful morphogenetic studies have been conducted in some plants of the family Scrophulariaceae such as *Mazus pumilus*, *Verbascum thapsus*, *Torenia fournieri*, *Antirrhinum majus* (Newbury, 1986; Atkinson *et al.* 1989, 1991), *Limnophila chinensis, Paulownia tomentosa, Digitalis purpurea, Digitalis lanata and Digitalis lutea, Digitalis obscura* (Arrillaga *et al.* 1986), *Angelonia salicariaefolia.* But nobody used anthers as explant for regenerating plants. However, androgenesis using anther cultures has been reported in a few species such as *Digitalis purpurea, Digitalis obscura* and *Lanaria macroccana* with the aim of producing haploid plants. Anthers of *Antirrhinum majus* exhibited indirect mode of organogenesis, i.e. shoot differentiated in subcultured anther derived calli on basal medium.

Similar mode of shoot development has also been noticed in anther cultures of *Digitalis purpurea* by Corduan and Spix (1975).

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