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Establishment of Regeneration Protocol for Canola (*Brassica napus* L.)

By Sabrina Shameen Alam, Laila Khaleda & Mohammad Al-Forkan

University of Chittagong, Bangladesh

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Establishment of Regeneration Protocol for Canola (*Brassica napus* L.)

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Abstract- The study was conducted with an aim of developing a genotype independent efficient *in vitro* regeneration system for Canola (*Brassica napus* L.) that is not grown in Bangladesh. Plant regeneration was achieved through embryogenic callus production from hypocotyls and cotyledonary leaves with petioles. The optimum medium for callus induction was found with 0.5 mgl^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D). The best shooting medium for canola contained 3.0 mgl-1 benzyl amino purine (BAP), 0.1 mgl^{-1} naphtalene acetic acid (NAA), and 5.0 mgl^{-1} $AgNO_3$ and produced maximum number of shoots when kinetin was used at a concentration of 0.5 mgl^{-1}. The profound positive effect of $AgNO_3$ for Canola was found for callogenesis at 0.5 mgl^{-1} and for regeneration at 5.0 mgl^{-1}. *In vitro* regenerated shoots of canola produced well developed root system on both the media with 2.5 mgl^{-1} NAA and 1.0 mgl^{-1} indole 3-butyric acid (IBA).

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I. Introduction

Canola is the oilseed crop of cold countries, specially, Canada, USA and Europe as cultivation of Canola requires cool temperature during growing season. Bangladesh has a remarkable demand for edible oil. Among the oilseed crops grown in this country, *Brassica* occupies first position in respect of area and production. In the year of 2003-04, the production was 2.11 lakhs metric ton (Mt), whereas the total oilseed production was 4.0 lakhs Mt (BBS, 2005). Bangladesh consumes around 1.73 million tons of oils per year of which about 1.6 million tons fully met up by import. Hence, it is necessarily important to increase the production of *Brassica* which can save the country from the huge import pressure. Among the *Brassica* species, canola is the most productive variety which is low in both erucic acid (<2% in the oil) and glucosinolates that differentiate it from earlier rapeseed varieties (Eskin and McDonald, 1991). However, this variety is not grown well in the country. So, it is necessarily important to introduce canola in Bangladesh for improving both dietary health and production.

One of the most challenging tasks of the plant breeders in this century is the production of crops with increased sustainability to fulfill the need of present and future in terms of both yield and quality. Though Conventional backcross technique is a very well established one for improving seed quality such as low erucic acid (Agnihotri *et. al.*, 2004), oil crop breeding is complex than breeding of cereals and legumes due to the requirement of simultaneous manipulation of different traits. Comparisons of traits between conventionally derived and genetically modified plants has been assessed (Ian and Adrian, 2003) and quality such as rooting efficiencies in *Agrobacterium*- mediated DNA transformed canola was seen to be increased (Cardoza and Stewart, 2003). However, breeders have developed varieties of canola that are shorter and more resistant to lodging and shattering (Salisbery and Wratten, 1999). The suitable time for harvesting local *Brassica sp.* in Bangladesh is very short and challenging. Hence, this study has been designed to establish an efficient *in vitro* regeneration system for Canola to utilize the *Agrobacterium*-mediated genetic transfer to introduce a user friendly Canola variety in Bangladesh.

II. Materials And Methods

Canola seeds were collected from University of Alberta, Canada. The explants collected from *in vitro* grown seedlings used for the experiments were: hypocotyls and cotyledons with petiole. MS (Murashige and Skoog, 1962) medium was prepared with 3% (w/v) sucrose and solidified with 0.4% (w/v) agar and was stored at 4°C. MS media was supplemented with different PGRs (Plant Growth Hormones) such as 2, 4-D, BAP and additives such as proline, casein hydrolysate (CH) and $AgNO_3$ in a different formulation to adopt with plantlets (callus, shoots, roots etc.) Plant materials were sterilized with 70% Ethanol followed by immersing in 0.1% $HgCl_2$. Explants were inoculated into callus induction media. For callus initiation from hypocotyls and cotyledons, MS basal medium was supplemented with 0.5, 1.0 and 2.0 mg l^{-1} of 2, 4 –D with or without $AgNO_3$ or proline or BAP alone or in combination at a concentration of 0.5 mg l^{-1}. After 28 days from the first inoculation, all embryogenic except non-embryogenic, brown, slow growth and rhizogenic calli were transferred to the regeneration media. The multiple shoots were separated from the calli after 20 days of shoot development and were transferred to separate jars containing respective 60 ml shooting media. Roots were initiated when shoots were placed on medium containing MS basal salts supplemented with 2.5 mg l^{-1} NAA, and 30 g l^{-1} (w/v) sucrose and semi-solidified with

Authors α σ ρ : Department of Genetic Engineering & Biotechnology, University of Chittagong, Chittagong-4331, Bangladesh.
e-mail: alforkancu@hotmail.com

0.4% agar. The plantlets with sufficient rooting system were taken out of the culture vessels and the roots were washed under tap water. The *in vitro* grown rooted plants were then transferred into small pots containing moisture soil. Hardening was carried out by periodical exposure of the plants to natural environment.

III. Results and Discussions

To avoid contamination of explants, Canola seeds were germinated on half strength of MS medium, 70% ethanol and 0.1 % $HgCl_2$ solution were used for sterilization. However, the use of 0.2% $HgCl_2$ was seen to reduce the percentage of seed germination, delayed the time required for germination and affected the elongation of the seedlings (data not shown). The effect of light on seed germination and seedling development were measured by using half strength of MS with different concentration of sucrose and agar without any growth regulators. Germination was seen to be better under dark, 100% in some cases. Callus initiated from Canola hypocotyls on medium supplemented with 0.5 mg l^{-1}, 2, 4-D, 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} $AgNO_3$, after 24 days of culture (Fig. 1.A.). Large callus was formed in that condition (Fig. 2. A.). However, no significant callus formed on MS medium supplemented with 0.5 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} BAP but without $AgNO_3$ (Fig. 2. B). Browning of explants and little or no callus formation was observed on MS medium supplemented with 0.5 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} $AgNO_3$ but without BAP (Fig. 2. C). Initiation of callus from Canola cotyledons was observed on the medium supplemented with 0.5 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} $AgNO_3$, after 24 days of culture (Fig. 1. B.). Using BAP on callogenesis was found to increase the size and percentage of calli formation (data not shown). Similar reports were provided by Ali *et. al.* (2007) and Khan *et. al.* (2002) that supports the present investigation. Again, the use of $AgNO_3$ was described to promote the growth of *Brassica* callus by Williums *et. al.* (1990). The effects of only IAA, and IAA and IBA in combination on callogenesis were also evaluated. In case of hypocotyls about 12% of the calli produced roots within 20 days of culture on medium containing IBA at 0.5 mg l^{-1}. In case of cotyledon, callus induction occurred within two weeks on both the medium and the induced calli were yellowish white. Whitish 1-2% of the cotyledonary explants produced roots in the medium containing IAA at 0.5 mg l^{-1} (Fig. not shown). To evaluate the effect of CH and proline independently on callus induction, BAP and $AgNO_3$ concentrations were kept constant, both at 0.5 mg l^{-1}. The callus was mostly green and overgrowth of callus was observed even when they were placed on shooting medium. Hypocotyls (100%) produced callus of embryogenic nature on this medium though the size of the callus were small and were dominant at the cutting edge of the explants (Fig. not shown). Callus production was better from hypocotyles than cotyledons on proline supplemented media in absence of $AgNO_3$, where the cotyledons swelled but produced very insignificant callus (Fig. 3).

Change in BAP concentration changed the frequency of shoot formation from cotyledonary and hypocotyl calli. Cotyledonary calli gave the best percentage of shoot induction and development (88.46%) when BAP concentration was 2.0 mg l^{-1}. Increasing BAP concentration to 2.5 and 3.0 mg l^{-1} lowered the shoot initiation potential of cotyledonary callus to 60% and 53.84%, respectively (Fig. 4). But increasing BAP concentration increased percentage of shoot production from hypocotyl calli from 53.46% to 75% (Fig. 4.). The initiation of shoots was earlier in cotyledonary calli (About 20% callli started shooting within 10-12 days).

To get a complete plantlet, the induction of root at the base of *in vitro* regenerated shoots is an indispensible step. Spontaneous root generation occurs sometimes on MS medium with hormonal supplements for the induction of shoots. These roots were not in direct connection with the developed shoots and they were developed from the calli placed on the regeneration medium. The plantlets with these roots were not found to be efficient to thrive in soil. Therefore, independent medium for the induction of roots from shoots were used in the experiment. The plant growth regulators (PGR) used for the purpose were NAA and IBA. Roots initiated and developed sufficient root system on medium containing 2.5 mg l^{-} NAA and 0.5 mg l^{-} BAP. Because of the root production efficiency of the calli in callus induction medium containing 0.5 mg l^{-1} IBA, this plant hormone was used in rooting medium at a concentration of 1.0 mg l^{-1}. Shoots produced efficient root system in this medium. The days required for root initiation also varied in different media (data not shown). However, finally, different stages of regeneration from canola callus were established in the study. The best initiation of shoots from hypocotyls was observed on regeneration medium supplemented with 0.5 mg l^{-1} Kinetin, 2.5 mg l^{-1} BAP and 5.0 mg l^{-1} $AgNO_3$ (Fig. 5. A.). Shoots elongated significantly after three weeks of inoculation (Fig. 5. B.). Shoots were developed from cotyledonary callus in the medium supplemented with 0.5 mg l^{-1} Kinetin, 2.5 mg l^{-1} BAP and 5.0 mg l^{-1} $AgNO_3$, (Fig. 5. C.) and canola plantlet were precisely regenerated in medium supplemented with 2.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 5.0 mg l^{-1} $AgNO_3$ (Fig. 5. D.)

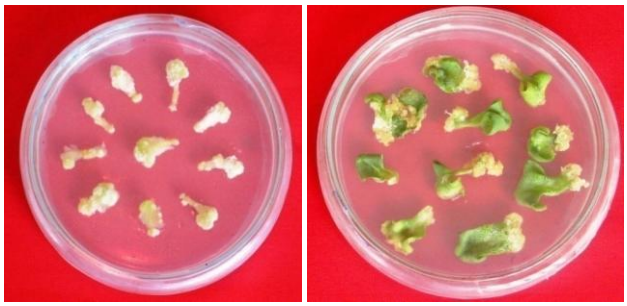

Fig. 1 : Calli grown from canola hypocotyls (A) and cotyledons (B) on medium supplemented with 0.5 mg l^{-1}, 2, 4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} $AgNO_3$, after 24 days of culture

Fig. 3 : Effect of proline on callus induction and development from hypocotyls (A) and cotyledons (B) of Canola, after 24 days

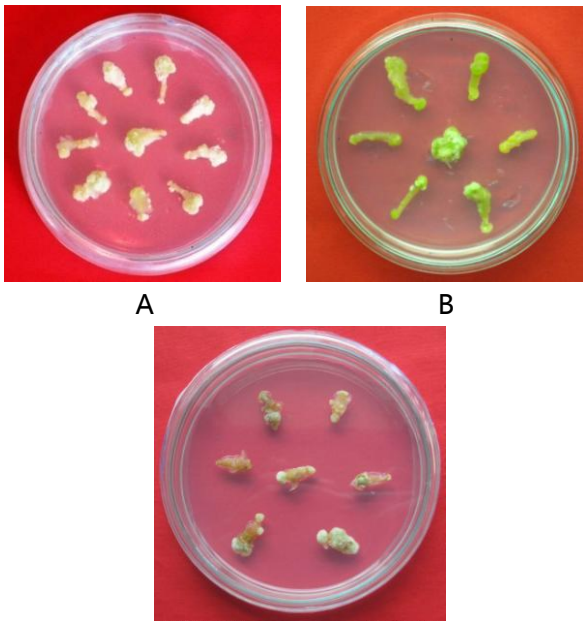

Fig. 2 : Effect of $AgNO_3$ and BAP on callus induction and development from hypocotyls of canola. Changes according to the discussion are shown from panel A to C

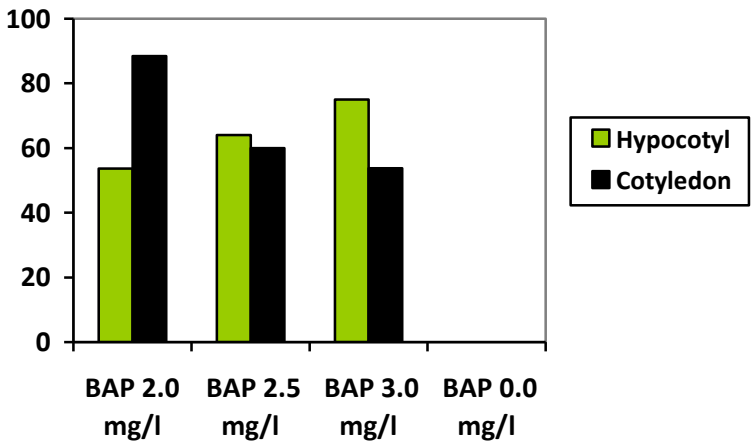

Fig. 4 : Changes in the percentage of shoot formation concentration changes shooting frequency from Canola on different media; changing BAP

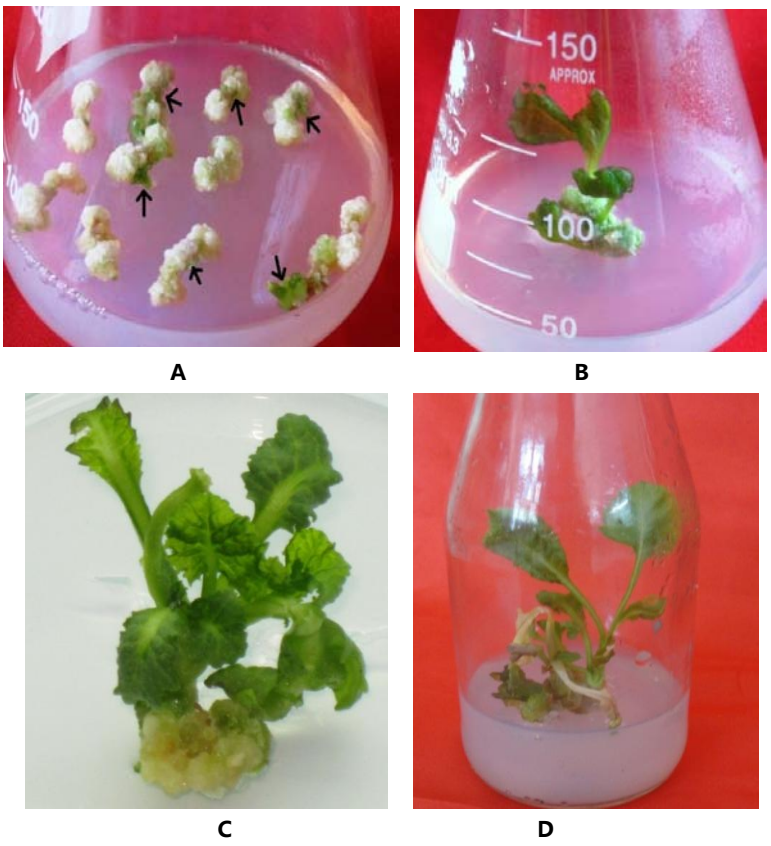

Fig. 5 : Different stages of regeneration from canola callus. (A) Initiation of shoots from Canola hypocotyls on regeneration medium supplemented with 0.5 mg l^{-1} Kinetin + 2.5 mg l^{-1} BAP + 5.0 mg l^{-1} $AgNO_3$, (B) Elongation of the shoots of Fig. A after three weeks of inoculation, (C) Development of shoots from Canola cotyledonary callus in the medium supplemented with 0.5 mg l^{-1} Kinetin + 2.5 mg l^{-1} BAP + 5.0 mg l^{-1} $AgNO_3$, and (D) Regenerated canola plantlet in medium supplemented with 2.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA + 5.0 mg l^{-1} $AgNO_3$

IV. Conclusion

A genotype independent efficient *in vitro* regeneration system for Canola (*Brassica napus* L.) has been established in the present study. Plant regeneration was achieved through callus production from hypocotyls and cotyledonary leaves with petioles. The optimum medium for callus induction and the best shooting medium has been described in the study. The positive effect of $AgNO_3$ for Canola was found for both callogenesis and regeneration. Cotyledonary calli were better in terms of shoot regeneration. *In vitro* regenerated shoots of canola produced rooting system on both the media with 2.5 mgl^{-1} NAA and 1.0 mgl^{-1} IBA.

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