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IN VITRO REGENERATION OF *BRASSICA NAPUS* L., CULTIVARS (STAR, CYCLONE AND WESTAR) FROM HYPOCOTYLS AND COTYLEDONARY LEAVES

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Abstract

Regeneration protocols were established for *Brassica napus* L., cultivars viz., Star, Cyclon and Westar. The conditions were optimized for callus induction, shoot and root regeneration. Seeds were surface sterilized using 70% alcohol, 0.01% $HgCl_2$ and few drops of Tween20 for ten minutes. Which reduced seed borne contamination. Callus induction ability was evaluated by using different concentration of 2, 4-D in combination with 0.5mg/L BAP and 0.5mg/L Silver nitrate, which was used for the first time in MS medium and efficient callus was produced using 0.5mg/L 2, 4-D. Silver nitrate in callus induction media resulted in green callus. Shoots were regenerated on callus using different concentrations of NAA with 2mg/L BAP and 5mg/L Silver nitrate in MS medium. 67-82% shoots were regenerated on media having 0.1mg/L NAA. The shoots were then rooted and best results were obtained on media having 0.3mg/L IBA in half strength MS medium.

Introduction

Canola (*Brassica napus* L.) is an important oilseed crop, ranking third only to soybean and palm oil in global production. Canola oil is widely used as cooking oil. Due to its lowest saturated fat content, it is appealing to health-conscious consumers (Ahmad *et al.*, 2002). In the Indo-Pakistan subcontinent, its per unit area production is three times less (665 kg ha^{-1}) as compared to the developed countries where its production is 2180 kg ha^{-1}. For better yield and resistance to diseases and insects it is necessary to analyze the architecture of *Brassica* genome and genome relationship among various species of the genus. Genome analysis and preferential pairing have extensively been utilized for characterizing *Brassica* genomes. Breeding system analysis, chromosome morphology, meiotic associations and molecular characterization indicated that *Brassica* is monophyletic in origin and it descended from an unknown six chromosomal prototype (Ahmad, 2001; Ahmad, *et al*., 2002). The agro-climatic conditions of many areas, especially the northern Pakistan are suitable for the production of Canola. Genetically improved seed of varieties in this regard are always constraints (Islam, *et al*, 2004, Ahmad & Hasnain, 2004). Meiotic analysis was carried out in M1 of pollen mother cells of genotype HS-98. The chromosome number was 2n = 38 (19 bivalent per cell), no univalent, multivalent and secondary association were observed. The pollen fertility percentage remained 93% of the 604 pollens observed. There are reports that the genotype exhibits both genetic and physiological stability (Islam *et al.*, 2006). Genetic modification of crop is rapidly becoming the technique of choice for the production of new agricultural varieties. There is need for efficient regeneration of plant in order to produce transgene of required characteristics.

Organogenesis is an indispensable tool for plant regeneration and transformation. The available information shows that regeneration through organogenesis has been accomplished from various tissues including cotyledons (Sharma *et al.,* 1990; Hachey *et al*., 1991; Ono *et al.,* 1994), hypocotyls (Yang *et al*., 1991), peduncle (Eapen and George 1997), leaves (Radke *et al.,* 1988), thin cell layers of epidermis and sub epidermis (Klimaszewska and Keller, 2002), roots (Xu *et al*., 1982), and protoplasts (Glimelius, 1984; Spangenberg *et al.,* 1986; Hu *et al*., 1999). However a hypocotyl remains the most desirable explants for tissue culture and has been used for *Brassica* regeneration. Experiments were carried out to study the establishment of an improved protocol for the efficient regeneration of *Brassica napus* from hypocotyl.

Materials and Methods

The study was carried out in the laboratory of Biotechnology, University of Malakand to standardize conditions for the *In vitro* regeneration of *Brassica napus* L. cultivars viz., Star, Westar and Cyclon from different types of explants i.e. hypocotyls and cotyledonary leaves.

Seeds were obtained from Agriculture Research Station North Swat and National Agriculture Research Center (NARC) Islamabad. The seeds were washed with a few drops of detergents in beakers and then placed for an hour in two layered nylon cloth for soaking in sterilized distilled water. The seeds were then submerged in 70% alcohol for half to one hour. The seeds were transferred to flasks containing 0.01 and 0.1 % $HgCl_2$ solution for 5-10 minutes and rinsed five times with sterile distilled water and transferred in culture plates containing half strength (MS) medium (Murashige & Skoog, 1962) with 5% sucrose and solidified with 0.8% agar, pH was adjusted at 5.8 and autoclaved at 15psi at $121C^0$ for 20 minutes. Physical conditions provided was 500 lux for 3 days followed by 2000 lux for 3-10 days, 16 hr light/8 hr dark cycle $23\pm1C^0$.

Cotyledonary leaves and hypocotyls were excised from 5-7 days old seedlings and cut into 0.5-1 cm pieces under laminar flow hood. The pieces were placed on MS medium supplemented with different concentrations of 2,4-D (Table 1) and 0.5 mg/l Benzylaminopurine and sliver nitrate. The callus induced was transferred to the shoot regeneration medium containing MS medium supplemented with different concentration of Benzylaminopurine, NAA and Sliver nitrate (Table 2). Shoot regeneration was carried out for both of the calli that derived form the hypocotyls shoots and cotyledonary leaves. For this purpose green and healthy portion of callus was taken and cut into pieces and inoculated on medium for shoot regeneration having several concentrations of hormones individually and in combination. Each experiment was conducted twice by raising 10-15 calli for each treatment. All the flasks were kept in growth chamber at 27^0C, 16h of photoperiod, and 60% relative humidity, to determine shoot regeneration potential of different calli at different hormone concentrations and combinations. Visual observations were taken after every three days and effect of different treatments was quantified on the basis of percentage of callus showing response for shoot regeneration.

Root regeneration was carried out both for the shoots that raised from the callus, previously regenerated from explants of hypocotyls shoots and cotyledonary leaves. For this purpose green and healthy shoots were taken and were placed on medium for root regeneration having several concentrations of hormones individually and in combination under the laminar air flow hood (Table 3). Each experiment was conducted twice by raising 1-5 shoots for each treatment.

Table 2. Percentage shoots formation ability from callus of various cultivars Star, Westar and Cyclon using different concentration of NAA and taking BAP (2mg/L), Silver Nitrat (5mg/L) constant.

NAA (mg/L)	Star (% age shoot formation)	Westar (% age shoot formation)	Cyclon (% age shoot formation)
0.0	0	0	0
0.1	82	67	78
0.2	55	45	23
0.3	40	34	34

Table 3. Percentage roots formation ability from shoots of various cultivars Star, Westar and Cyclon using different concentration of IBA.

Concentration of IBA	Star (% age root formation)	Westar (% age root formation)	Cyclon (%age root formation)
0.0	13	25	21
0.1	45	65	34
0.3	87	90	89
0.5	67	76	65
0.7	54	35	45

Results

Callus induction, shoot regeneration and root formation was successfully carried out in all the three selected cultivars of *Brassica napus* L. (Star, Westar and Cyclone). It was found that 0.5mg/L 2,4-D gave best results with 95-96% callus induction with maximum weight in cultivar Star in both hypocotyle and cotyledonary leaves while cultivar Westar showed best results of 96-98% and Cyclon 94-96% in same concentration of 2,4-D taking BAP and Silver nitrate @ 0.5mg/L (Table 1).

Shoots formed in all cultivar of *Brassica napus* L. were rooted using half MS medium supplemented with various concentration of Indole Butaric Acid. Best results were obtained at 0.3mg/L IBA. Star resulted in 87% roots formation in all shoots previously regenerated while in Westar 90% root formation was recorded and Cyclon showed 89% root formation.

Discussion

The germinating seeds were used as a source of explants for callus induction. The explants were inoculated on MS medium with variable range of 2, 4-D keeping the concentration of BAP and Silver nitrate as constant following the standard as reported by Cardoza & Stewart (2003). The callus was induced in almost four out of five treatments. There was no production of calli in the absence of 2, 4-D. The same results with no calli on 0%, 2, 4-D have been reported by Khan *et al.*, (2002). The callus was induced by using 0.5mg/L of 2, 4-D, Sliver nitrate and BAP, where the green calli with more weight was produced. Same results have been obtained by Stewart & Cardoza (2003) who found best results of callus induction using 1mg/L 2, 4-D with 0.5mg/L BAP. Khan *et al.*, (2002) reported 2mg/L as best calli producing concentration using only 2, 4-D for the callus induction in *Brassica napus* L. cultivar Oscar. Qain & Zhang (2004) reported best callus using 1.5mg/L 2, 4-D without using $AgNO_3$ for callus induction. $AgNO_3$ is considered as ethylene inhibitor (Tang *et al*., 2003) and is reported to be used in shoot

induction medium in *Brassica napus* L., Cultivar Westar (Cardoza & Stewart, 2003). In the present study $AgNO_3$ has been used for the first time in callus induction and best results have been obtained for callus induction.

Fig. 1. Callus of cultivars: A, Star; B, Westar; C, Cyclon.

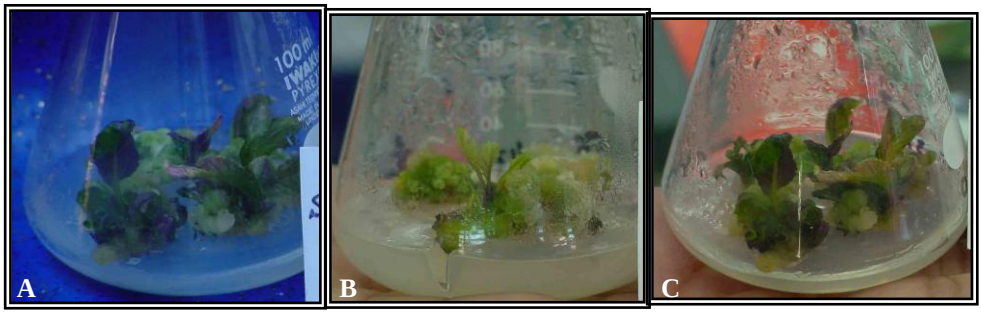

Fig. 2. Shoots of cultivars: A, Star; B, Westar; C, Cyclon.

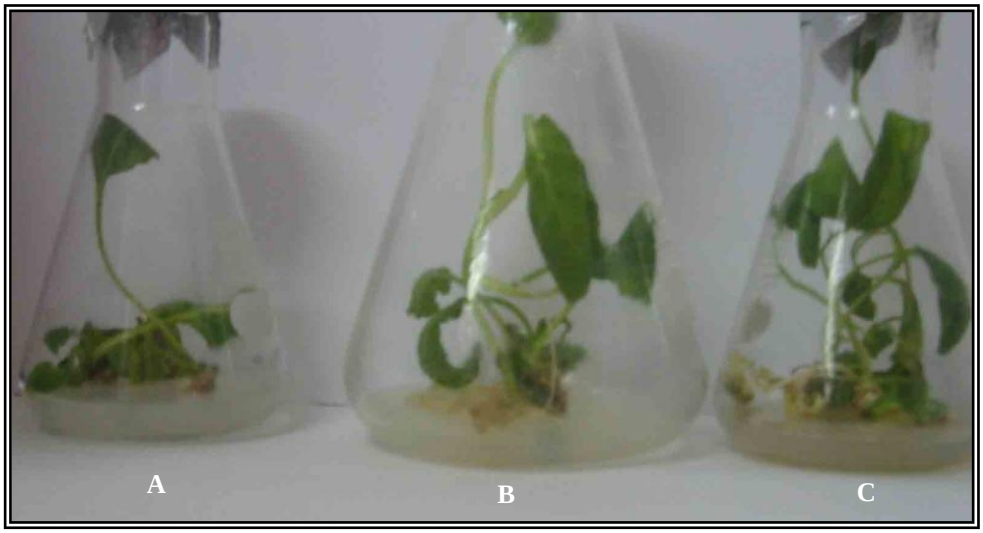

Fig. 3. Complete regeneration of the three cultivars: A, Star; B, Westar; C, Cyclon of *Brassica napus* L.

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