Enhanced rooting and regeneration of canola (*Brassica napus* L.) cultivars after a brief period (shock) on growth regulator-free Murashige and Skoog (MS) medium

The comparative organogenesis of *Brassica napus* L cultivars Cyclone, Star and Westar was studied. The cotyledonary explants gave a higher response to all the combinations of 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and benzylamino purine (BAP; 0.5, 1.0,1.5 and 2.0 mg/L) used for optimizing the conditions for callus induction. The best mean weight and mean length of callus was obtained at 0.5 mg/L 2,4-D and 1.5 mg/L BAP for Star cotyledonary explants. For the increased rooting and thus complete plant regeneration, a shock or a new method of exposing the explants culture to growth regulator free medium for seven days was performed followed for shooting on MS medium with growth regulators. The method was applicable to both hypocotyl and cotyledonary explants. The Shoot Induction Frequency for hypocotyl (6-34%) in the three cultivars is higher than the cotyledonary explants (3-23%). The method is speedy and almost all the shoots and some unshooted calli (78%) form roots on the same media without prior transfer to rooting medium. The analysis of variance (.05) showed that the data is significantly different, and all the variation is due to the different groups/sources in the experiments. Thus, it is recommended to use separate explants of each variety for gene transformation, especially hypocotyl explants of Westar.

Key words: Tissue culture, regeneration, *Brassica napus*, hypocotyle, cotyledon.

INTRODUCTION

The genus *Brassica* includes some of the very important crop species (Knutzon et al., 1992; Assou, 2023; Dawud et al., 2024) and is one of the most economically important genera in the Brassicaceae family (syn. Cruciferae). The Brassicaceae family comprises about 3000 species (Neeser et al., 1999). *Brassica* vegetables contain little fat, and are sources of vitamins, minerals, and fiber. They also contain a large number of novel

phytochemicals, some of which protect against carcinogenesis (Willcox et al., 2003). *Brassica napus* ranks the third among the oil crops, following palm oil and Soya oil and the fifth among economically important crops, following rice, wheat, maize and cotton (Cardoza and Stewart, 2003). The seed contains up to 45% of edible semi-drying oil. It is also used as a luminant or lubricant in soap making (Greville, 2005). Canola is the Canadian oil association trademark which commonly refers to oil seed rape or any rapeseed, which is most often *B. napus* (Cardoza and Stewart, 2004b) with less than 2% of erucic acid (C22:1) in the oil and less than 30 µmol of any one or all of the four major aliphatic glucosinolates named as 3-butenyle glucosinolate, 4-pentenyle glucosinolate, 2-hydroxy-3 butenyle glucosinolate and 2-hydroxy-4-pentenyle glucosinolates per gram of air dry oil free solid (Friedt and Luhs,1998; Katavic et al., 2001). Plant cell and tissue culture, also referred to as *in vitro*, axenic, or sterile culture is an important tool in both basic and applied studies and commercial application (Thorpe, 1990). The regulatory factors in the culture medium that regulate organogenesis (Thorpe, 1993) include both naturally occurring and synthetic plant growth substances, as well as various environmental stimuli (Lakshmanan et al., 1997). With the increasing demand for canola oil, genetic engineering which reduces the time to develop a new variety has replaced conventional breeding and this technology mainly depends on tissue culture techniques (Hazrat et al., 2007; Maheshwari et al., 2011).

Plant tissue culture technology has been successfully used for the commercial production of pathogen-free plants and to conserve the germplasm of rare and endangered species (Fay, 1992). Canola might also be especially useful as a vehicle to overproduce pharmaceutically active proteins and edible vaccines by applying the genetic engineering technology (Giddings et al., 2000; Moghaieb et al., 2006).

MATERIALS AND METHODS

Plant

The hypocotyl and cotyledonary explants of three *B. napus* canola cultivars namely Cyclone, Star and Westar were used. The seeds of cultivar Cyclone were obtained from the National Agriculture Research Center (NARC) Islamabad while the seeds of cultivars Star and Westar were obtained from the University of Agriculture, north Swat, Khyber Pakhtunkhwah. The dark healthy seeds were selected and surface sterilized (Farooq et al., 2019).

Seed surface sterilization and germination

The required number of seeds of the *B. napus*, cultivar Cyclone were first washed by submerging them in water for 1 h to remove the dust. The seeds were transferred to 70% ethanol for 1 min followed by immersion in 0.01% (w/v) mercuric chloride for 1 min. A few drops of Tween 20 were added as a surfactant and wetting agent to the mercuric chloride solution. Then the surface sterilized seeds (Naghisharifi et al., 2024) were transferred to autoclaved distilled water and rinsed 2-3 times. The seeds were germinated in Petri plates on 0.8% agar (w/v). Then, incubated at 25±2°C in complete dark. After 2 days the seeds were transferred to a 16/8 h day/night photoperiodic regime under cool white, fluorescent lights (1000-Lux) for 5 days (Ali et al., 2007).

Explants preparation

Cotyledons and hypocotyls segments from 7 days old seedlings were used as explants. These Hypocotyls 1-3 mm explants were carefully excised from the seedlings without including any of the meristematic axillary buds. About 25 to 30 seedlings per cultivar were used. The explants were readily used for different manipulations using the Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) modified with various growth regulators.

Complete plant regeneration

For callus induction the MS medium was modified with 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L benzylamino purine (BAP) with 12 replicas for each of the hypocotyl explants of each variety and 8 explants per flask. All the pre-sterilized flasks were plugged with the sterilized cotton, labeled and incubated at 25±2°C under a 16/8 h day/night photoperiodic regime (1000-Lux) for 14 days and then all the explants were transferred to shock medium. A new method of brief shock to the explants was introduced in which all the surviving and uncontaminated explants induced for callus were transferred to simple MS medium (Table 1) solidified with 8 g/L agar without any growth regulators to provide a brief shock to the explants and kept in the growth chamber conditions for 7 days then transferred to shooting medium. The shooting medium was prepared by modifying the MS medium with 0.1 mg/L 1-naphthaleneacetic (NAA) and 2 mg/L BAP to culture all the surviving explants from the shock media. After 21 days the explants were subcultured on the same shooting medium. The visual observations were taken on weekly basis continuously. The shoots were cut from the calli obtained from the shooting media through a sterilized razor blade and transferred to the media having half strength MS salts with 10 mg/L sucrose, 1.2 g/L agar and 0.5 mg/L IBA were used to induce rooting in the calli. The adjuvant $AgNO_3$ is a prerequisite in all *B. napus* tissue culture media (Akasaka-Kennedy et al., 2005). The shooted calli regenerating roots thus give rise to complete plants having both shoots and roots. The number of plants producing shoots and total shoots were counted. The Callus Induction Frequency (CIF), Shoot Induction Frequency (SIF), Root Induction Frequency (RIF) and Plant Regeneration Frequency (PRF) were calculated by the formulae (Moghaieb et al., 2006). The Mean, Standard Error of Mean (SEM), range and Standard Deviation (SD) for the average shoot number were confirmed by the Graphpad PRISM and SPSS Software. The regenerated plantlets were successfully acclimatized and transferred to glass house (Hussain et al., 2014).

RESULTS AND DISCUSSION

In the present study, media with different combinations of Auxin (2,4-D) to Cytokinin (BAP) were used to induce callus in the hypocotyl and cotyledonary explants of the *B. napus* L. cv Cyclone, Star and Westar. The study

Table 1. The callus, shoot, root, and complete plant regeneration from hypocotyl and cotyledonary explants of *Brassica napus* L cv Cyclone, Star, and Westar at 28th day on shooting media.

Plant		Variety					
		Cyclone		Star		Westar	
		Hypo	Coty	Hypo	Coty	Hypo	Coty
Total Explant No.		80	79	94	90	92	95
Callus inducing explants (14 days)	No	79	87	87	85	90	93
	%	99	99	92.5	94.4	97.8	97.9
Shock on growth regulator free medium for 7 days							
Shoot initiations (28th day)	No	5.0	20	32	14	9	3
	%	6	23	34	15.5	9.8	3.2
Roots inducing explants (28th day)	No	59	60	74	80	58	57
	%	73.7	68	79	89	63	60
Number of shoots	*Range	1-5	3-9	1-9	1-6	1-8	2-4
	Mean ± SEM	2±1	5.6±0.84	4.8±0.70	3.5±0.885	3.14±0.857	3±1
	S.D	2	2.387	2.443	2.168	2.268	1.414

*Range shows minimum and maximum values. *Hypo shows hypocotyl and coty shows cotyledon.

showed that high percentage (92-99%) formed callus (Al Ramadan et al., 2021). There was no significant difference between the different explants of a cultivar and among the cultivars for callus induction ($P<0.05$). The cotyledonary explants generally gave calli with more mean weight and mean length. The same results were obtained by Zhang and Bhalla (1999). They used BAP, NAA and gibberellic acid (3) in the study of seven commercial Australian cultivars of oilseed *B. napus* seedlings obtaining high callus induction (85-100%). The results are in agreement with Moghaieb et al. (2006) who obtained high 99-100% CIF using MS salts with B5 Vitamins and 1 mg/L 2,4-D, later on transferred it to shooting media. According to the review of Cardoza and Stewart (2004a) the hypocotyl segments were the most desirable for plant tissue culture and hence been used for most *Brassica* species because of their ability to regenerate. Khan et al. (2002a) has used the upper and lower portions of hypocotyl explants (Figure 1). In the present study generally the cotyledonary explants showed more CIF (Figure 2) and produced calli (Figure 3) with more mean weight and mean length. The findings of Stewart et al. (1996) as well as Cardoza and Stewart (2003) showed a lower callus induction for Canola cultivar Westar. In the first case the main reason for the lower callus induction was hyperhydration. Hyperhydration can retard the growth of the Westar tissues which was possibly traced to occur due to the gelling agent concentration. The problem in the present study occurred but at later stages when the Westar Callus was just starting shoots initiations. Therefore, this may have reduced the percentage of shoot regeneration. The hyperhydration can also occur due to high cytokinin level, high temperature, and type of the culture vessel. In such stresses there is more water retention and plants take up more water. The percentage of explants forming shoots varied greatly between the *B. napus* L. cultivars Cyclone, Star and Westar as well as the different explants (hypocotyl and cotyledon) on MS media with the Auxin (NAA) to Cytokinin (BAP) ratio. These findings exhibited a varied response (3-34%) to shoot regeneration (Figure 4) of different explants from the cultivars. The Shoot Regeneration Frequency (SRF) is 34% for Star hypocotyl explants and 15.5% for Star cotyledonary explants (Table 1). The findings of Zhang and Bhalla (1999) show that one of the *B. napus* cultivars named RK-7 had a low shoot regeneration (18%) from cotyledonary explants while the same cultivar had a higher (27%) shoot regeneration from the hypocotyl explants thus it is clearly shown that the regeneration depend on explants type and genotype. The average shoot number for the hypocotyl and cotyledonary explants of the cultivars Star, Westar and Cyclone in the present study varied from 2 to 5.6. Zhang and Bhalla (1999) reported a lower average shoot number range (1.13-2.55). Jain et al. (1988) reported average shoot number of shoots per cotyledonary explant varied from 0 to as many as 50 in some *Brassica* species. Khan et al. (2002b) obtained 92% regeneration for cyclone using NAA and BAP along with other adjuvants as gibberellic acid and morpholinoethane sulfonic acid (MES) using the upper portions of the hypocotyl and 97% regeneration from the lower portion of the hypocotyl by the same method for Canola cultivar Dunkled. Khan et al. (2003) obtained the same results for

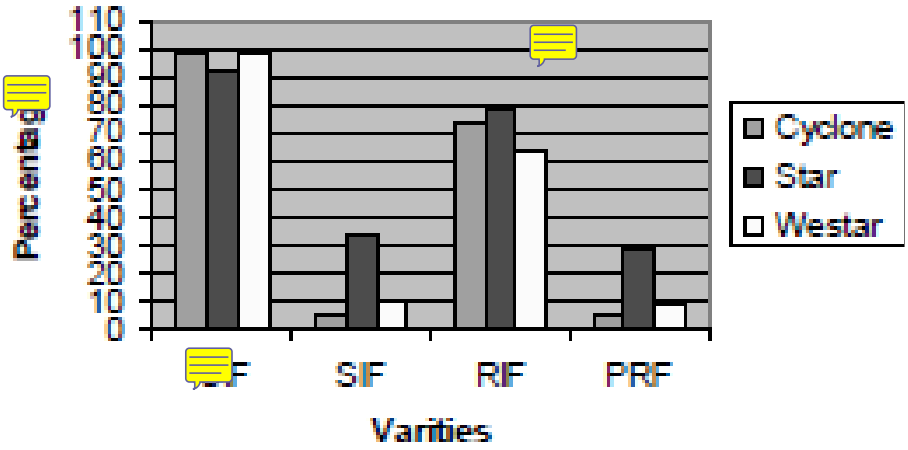

Figure 1. Comparison of the different characters of Hypocotyle explants.

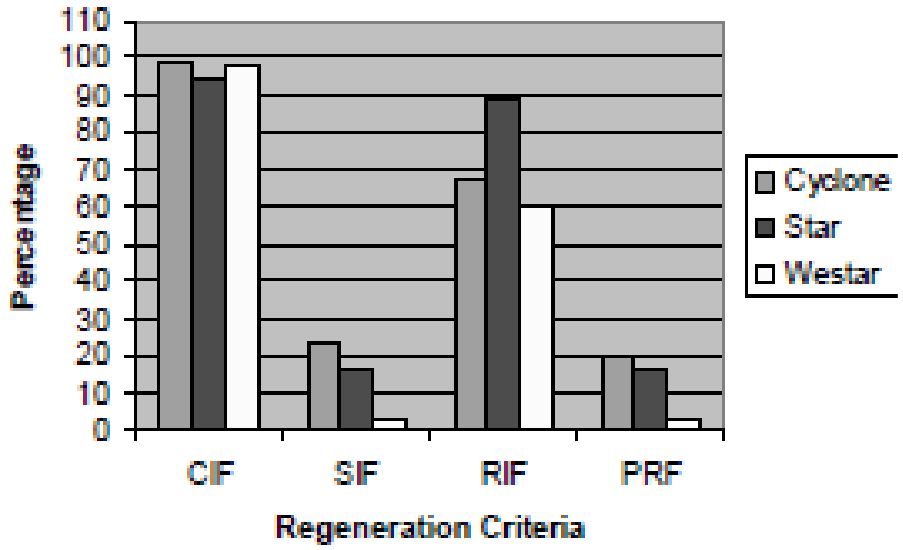

Figure 2. Comparison of the different characters of cotyledonary explants.

Cyclone. The low percentage of shoot regeneration in the present study may be due to the combination of the Auxin (NAA) and Cytokinin (BAP). The results reported by Jain et al. (1988) show that BAP in combination with NAA yielded no or a reduced number of shoots. The Westar has a reduced SRF (9%) for hypocotyl explants and 3% for cotyledonary explants as evident from Table 1. Cardoza and Stewart (2003) reported 16.9% regeneration for the cultivar Westar after a 6.4% loss with hyperhydration, but the hormonal regime was preconditioned with 1 mg/L 2,4-D followed by 4 mg/L BAP and 2 mg/L Zeatine. The present study showed that Shoot Regeneration Frequency (SRF) was 20% for Cyclone cotyledonary explants while 5% for the Cyclone hypocotyl explants. The rate of regeneration was slower with the hypocotyl explants as compared to cotyledons. The same results have been reported by Khehra and Mathias (1992) that the important factors for shoot regeneration were explant type and genotype and the influence of hormone regime was negligible. In the present study, cyclone has a lower (5%) regeneration in hypocotyl explant as compared to the 15.5% from its cotyledonary explants; this is in agreement with Irwin et al. (1999). According to Phogat et al. (2000), *B. napus* cultivar GSL-1 showed better regeneration efficiency than Westar. It means that regeneration is genotype specific. According to the review by Cardoza and Stewart (2004a) genotype is a limiting factor that severely limits the germplasm that can be manipulated or improved. Regeneration also depends on the age of the explants. Young explants gave better results than older explants. In *B. napus* 4-day old seedling explants yielded optimal

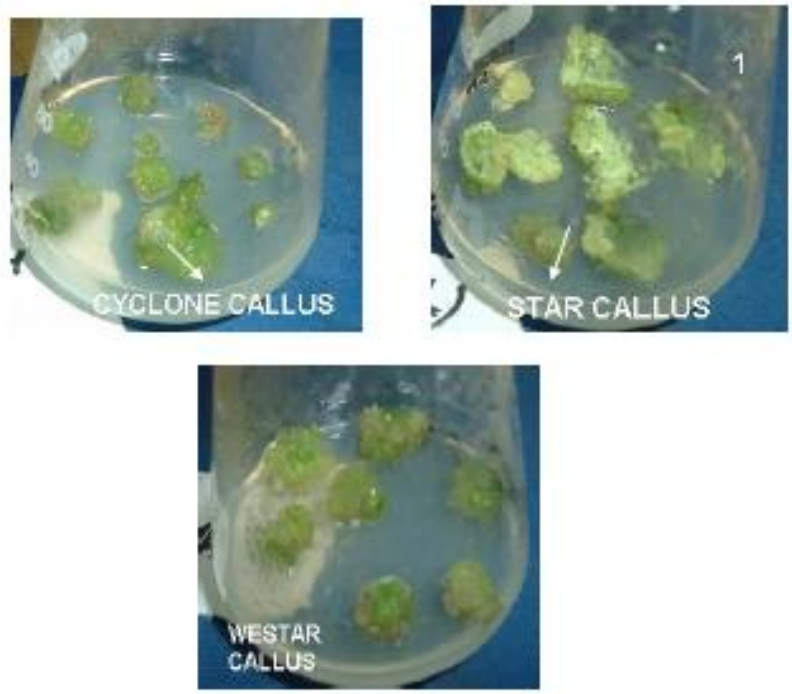

Figure 3. Calli of *B. napus* cv Cyclone Star and Westar.

Figure 4. New shoot initiation from the callus and complete regenerated plantlets with both shoots and roots.

regeneration of 90% as stated by Ono et al. (1994). The regeneration kept on decreasing when the age of explant increased above 4 days. He also reported that there was a huge variation from 0 to 90% in the 100 cultivars tested. Jin et al. (2000) reported in cabbage explant of 2-week-old seedling gave optimal results. Xiang et al. (2000) used hypocotyl explants of 5-day-old seedlings under different combinations of BAP (2,4,6 mg/L) and NAA (1,2,3 mg/L) with silver nitrate for *Brassica campestris* subsp *parachinensis* and found the best results at 4.0 mg/L BAP and 2.0 mg/L NAA. While explants younger or older resulted in lower shoot regeneration. Stewart et al. (1996) obtained shoot regeneration for cultivar Westar almost similar to the present results. The root induction (0.277%) is very low than the root induction for Westar (58%) obtained from the present study. The high root regeneration frequency may be due to the use of basal part of the hypocotyl which is also reported by Slesak et al. (2005) who obtained rhizogenesis for hypocotyls (98-100%) and cotyledons (54-85%) of cultured *in vitro* in *B. napus* L., cv. Kana. Short treatment (1 and 3 days) through MS media having 2,4-D and then transferred to hormone free medium obtaining adventitious shoots with the highest frequency (14% of explants) on hypocotyls

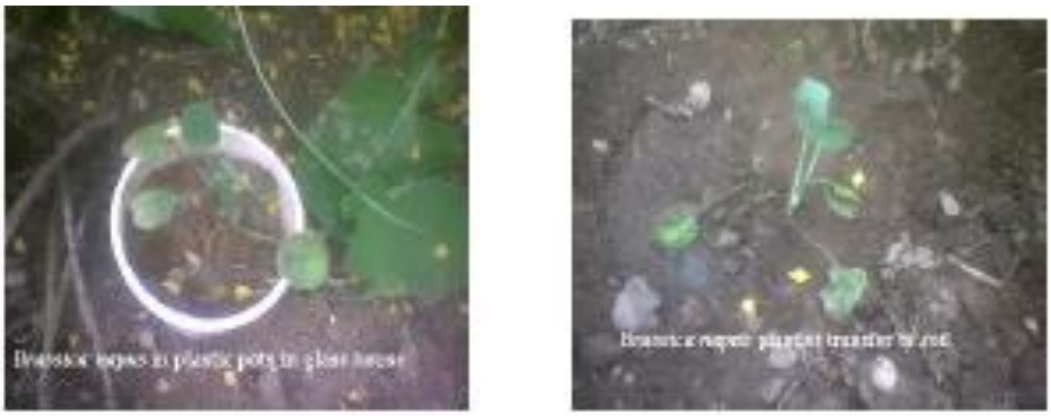

Figure 5. Acclimatization and transfer to glass house.

cultured. Histological analysis clearly indicated that the basal part of hypocotyls was involved in root formation and callus production, and the apical part for shoots. Similar method was used by Filek et al. (2005) who reported more calli than untreated controls when the upper apical part of the hypocotyl was facing the cathode of the electric field. The hypocotyle segments from upper part of rape (*B. napus* L., cv. Goczanski) hypocotyls were stimulated by different combinations of voltage/time. Based on these results they suggested that electric field action can be connected with its influence on specific concentration of oxidative substances and hormone distribution in cells. In contrast to changes in fresh weight, electric field treatment (30 V/30 s) stimulated a higher accumulation of 2,4-D and BAP in basal parts of hypocotyls than in apical ones. Damgaard and Rasmussen (1991) reported clones of hairy root formation which were subcultured on hormone free liquid MS medium but the results are not available. Julliard et al. (1992) compared the regeneration abilities of the *in vitro* cultured explants on media supplemented with several plant growth regulator combinations. There was no regeneration on hormone free media. The method is different from the present study in the sense that hormone free medium cannot be permanently used but for a brief period of seven days only (Khan et al., 2022; Imran et al., 2023).

Acclimatization and transfer to glass house

The calli having both shoots and roots were washed off the media from their roots completely and transferred in five-inch plastic pots (Figure 5) using the available potting mix and sphagnum peat moss. The pots were covered with plastic dome to retain humidity and transferred to glasshouse conditions. In the glass house the plastic dome was removed off the pots and plantlets were let to acclimatize the glass house conditions. After acclimatization the plantlets were successfully transferred to the soil.

Conclusion

The results of this study indicate that a brief shock treatment can significantly enhance the rooting and regeneration potential of Canola cultivars when cultured on growth regulator-free MS medium for seven days. This alternative approach may have implications for reducing the reliance on exogenous growth regulators in tissue culture protocols for Canola and other crops. Further research is needed to elucidate the underlying molecular mechanisms and optimize the shock treatment conditions for different Canola genotypes.

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