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IN VITRO ANALYSIS OF CALLUS INDUCTION IN INTERSPECIFICALLY HYBRIDIZED F_{4-5} POPULATIONS OF *BRASSICA*

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Abstract

Explants of F_{4-5} populations of 3 *B. napus* (A-20-80, Maluku and Dunkled) interspecifically hybridized with 3 *B. campestris* lines (1203, 2163 and 2065) were cultured on MS-medium + 2, 4- D (0.5 mg l^{-1}) + Kinetin (0.25 mg l^{-1}) for *In vitro* analysis. On average, frequency of callus induction was better in F_{4-5} populations and *B. napus* species than *B. campestris* for seed, cotyledonary and leaflet explants. The *B. napus* parental lines were better than F_{4-5} population in inducing callus in explants of leaflets and cotyledons. The callus induction was the earliest in F_{4-5} population as compared to both *B. napus* and *B. campestris* species. Callus induction in seed explants of *B. napus* was late, while early in leaflet and cotyledonary explants as compared to *B. campestris*.

Introduction

The rapeseed oil *Brassica* species (*B. napus*, *B. rapa* and *B. juncea*) are among the most important vegetable oil and protein-rich meal crops in the world. During last 30 years world production of rapeseed oil *brassica* showed upward growth with 14% share of the world's vegetable oils. *Brassica* species are the third leading source of vegetable oil of the world after soybean and oil palm, and also the world's second leading source of protein meal after soybean (Anon., 2010). This success is largely attributed to continuous and intensive breeding efforts (Zhou, 2001). Many *brassica* species show a high degree of relatedness, which allows crossing to occur across species and even genera. Several breeders and geneticists used interspecific hybridization for the genetic improvement of rapeseed oil brassica crop (Bothmer *et al*., 1995; Davey, 1939; Honma & Summers, 1976; Yarnell, 1956). *B. compestris* shares the A genome with *B. napus* and researchers have studied the gene transfer between these species in both directions (Bing *et al*., 1996; Jorgensen *et al*., 1996; Ellstrand *et al*., 1999; Wilkinson *et al*., 2000).

In Pakistan, the critical shortage of edible oil is adversely affecting our economy and more than 75% of the total foreign exchange allocated for the import of food items is used for the purchase of edible oil. Presently domestic production of oil meets only 30% of the country's requirements. After cotton, rapeseed-mustard is second most important source of oil in Pakistan. *B. napus* (Gobhi Sarsoon) is an important rapeseed-mustard crop in the world as well as in Pakistan (Munir *et al*., 2008).

Rapeseed or *Brassica napus* L. was the first crop species in which breeding was achieved by both traditional as well as modern methods (Burbulis *et al*., 2005). Therefore, *B. napus* has become an object of extensive tissue culture studies and breeding. Tissue culture is a process whereby small pieces of living tissues (explants) are isolated and grown on semi-refined medium aseptically. The explant may be as large as a seedling or an organ such as ovule, embryo etc., and as small as single cell or protoplast. Success in plant tissue culture is usually based choice of an explant and culture medium used (Munir *et al*., 2008). A range of plant tissues have been used for regeneration of B. napus shoots, including cotyledons (Narasimhulu *et al*., 1989), and stem and leaf segments of one-month old seedlings (Ovesna *et al*., 1993). Cell and tissue culture involving variability and selection efficiency are two indispensable components of molecular breeding (Lichtenstein & Draper, 1985). Therefore *In vitro* culture could serve as the best method for comparison of different plants through different explants.

The aim of the present paper was to compare the performance of F4-5 populations of interspecifically hybridized *B. napus* and *B. campestris* with their respective parental lines by *In vitro* culture.

Materials and Methods

Plant material: Three parental lines (i.e. A-20-28, Maluko and Dunkled) of *Brassica napus* (*Bn*) were crossed with three parental lines (i.e. 2065, 2163 and 1203) of *Brassica campestris* (*Bc*). Selection was made in F_2 and the selected materials were advanced in Khyber Pakhtunkhwa Agricultural University Peshawar till F_4. Four F_4 derived F_5 (F_{4-5}) populations (*Bn* x *Bc*) were reselected based on their field performance to analyze their *In vitro* response for callus induction (Table 1).

Table 1. Six parental lines and their interspecific F_{4-5} populations used for *In vitro* culture.

Parent lines 1	Origin	Parental lines 2	Source	F_{4-5} Populations
Brassica napus (*Bn*)		*Brassica campestris* (*Bc*)		All *B n* × *B c*
A-20-28	unknown	1203	Mingora	A-20-28 × 1203
Maluku	Australia	2163	Mingora	Dunkled × 1203
Dunkled	Australia	2065	Mingora	Maluku × 2065
				Maluku × 2163

Basal medium: Murashige & Skoog -MS (1962) basal medium supplemented with 2, 4-D (0.5 mg l^{-1}) and Kinetin (0.25 mg l^{-1}), 3% sucrose and solidified with 0.8% Difco-Bacto agar, were corked and sealed with aluminum foil and autoclaved at 121°C for 20 min afterwards. The MS medium was adjusted to pH 5.6 or 5.7 before adding agar and autoclaving.

Seed culture: Seeds from selected interspecifically hybridized F_4 derived F_5 (F_{4-5}) populations along with their respective parental lines were surface sterilized for 5 to 10 seconds in 70% Ethanol solution in universal bottles. Immediately after, seed were washed with sterilized water 3 to 5 times. In the next step, seeds were shifted to 70% Sodium hypo-chloride (NaOCl) solution with continuous shaking for 10-15 min and were washed again for 3 to 5 times with sterilized water. 20ml test tubes containing 10ml of MS basal medium supplemented with 2, 4-D (0.5 mg l^{-1}) and Kinetin (0.25 mg l^{-1}), 3% sucrose and solidified with 0.8% Difco-Bacto agar, were corked and sealed with aluminum foil and autoclaved at 121°C for 20 min afterwards. The MS medium was adjusted to pH 5.6 or 5.7 before adding agar and autoclaving. Seeds were aseptically cultured in 20ml test tubes containing 10ml MS-medium after autoclaving by standard culturing technique for callus induction (Fig. 1a). The experiment was repeated 4 times.

Production of donor plants: Seed and 1 week old plantlets (grown separately, and excised aseptically) from all F_{4-5} populations and their respective parental lines were cultured on callus inducing medium in test tubes (Fig. 2a). Test tubes were corked and sealed with aluminum foil and incubated at 22ºC with 16-h photoperiod to produce donor plants for excision of cotyledonary and leaflet explants.

Leaflet explants: Leaflet explants from 2-week-old plantlets were removed in similar fashion and were aseptically cultured in 20ml test tubes containing medium with similar composition to cotyledonary explants (Fig. 3a). The medium was autoclaved on 121ºC for 20 min and pH was set to 5.7 before culturing the leaflets. The experiment was replicated four times.

Callus induction frequency [$CIF_{(S)}$]: Seed explants inducing callus was calculated as percent $CIF_{(S)}$, by using the following formula. Data was converted to mean CIF (s) (Table 2).

$$CIF_{(S)} = \frac{\text{Number calli producing explants (seed)}}{\text{Total number explants in the culture}} \times 100$$

Callus induction interval (CII): CII was calculated as days to callus induction taken by cotyledonary explants. Data was converted to means (Table 3).

Callus Induction Frequency [$CIF_{(L)}$]: CIF for leaflet explants $_{(L)}$ were calculated as the percent leaflet explants inducing callus by using the following equation and was converted to mean CIF (L)] (Table 2).

$$CIF_{(L)} = \frac{\text{Number calli producing explants (leaflets)}}{\text{Total number explants in the culture}} \times 100$$

Table 2. Mean values for callus induction frequencies (%) for different explants of four F_{4-5} populations and their respective parental lines.

F_{4-5} populations	Type (*Bn* x *Bc*)	Seed explants	Cotyledonary explants	Leaflet explants
1	A-20-28 × 1203	83.30	97.90	66.67
2	Dunkled × 1203	77.80	78.70	79.90
3	Maluku × 2065	78.90	84.60	81.67
4	Maluku × 2163	88.90	86.67	88.00
Means		82.23	86.97	79.06
Parental genotypes of *Brassica napus*				
1	A-20-28	66.67	99.00	83.30
2	Maluku	94.40	80.67	83.30
3	Dunkled	83.3	98.00	88.00
Means		81.46	92.56	84.87
Parental genotypes of *Brassica campestris*				
1	1203	77.67	80.33	83.33
2	2163	77.80	76.67	80.67
3	2065	66.70	70.20	72.20
Means		74.06	75.73	78.73

Table 3. Mean callus induction interval (%) for different explants of four F_{4-5} populations and their respective parental lines.

F_{4-5} populations	Type (*Bn* x *Bc*)	Seed explants	Cotyledonary explants	Leaflet explants
1	A-20-28 × 1203	37.56	11.78	13.67
2	Dunkled × 1203	45.11	11.56	13.00
3	Maluku × 2065	54.67	11.67	15.56
4	Maluku × 2163	34.44	13.50	13.44
Means		42.95	12.13	13.92
Parental genotypes of *Brassica napus*				
1	A-20-28	51.22	12.67	13.78
2	Maluko	51.00	12.33	14.89
3	Dunkled	52.67	12.44	14.56
Mean		51.63	12.48	14.41
Parental genotypes of *Brassica campestris*				
1	1203	47.11	12.89	14.33
2	2163	45.22	14.22	14.33
3	2065	57.67	14.89	18.67
Mean		50.00	14.00	15.78

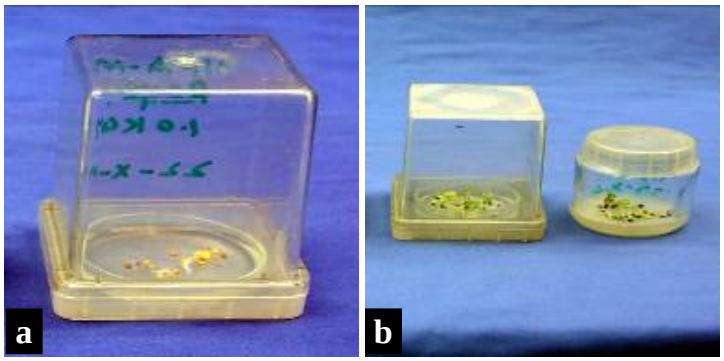

Fig. 1. Callus induction of Seed Culture: (a) Seed cultured on MS-solidified medium, (b) Young seedling emerging from seeds

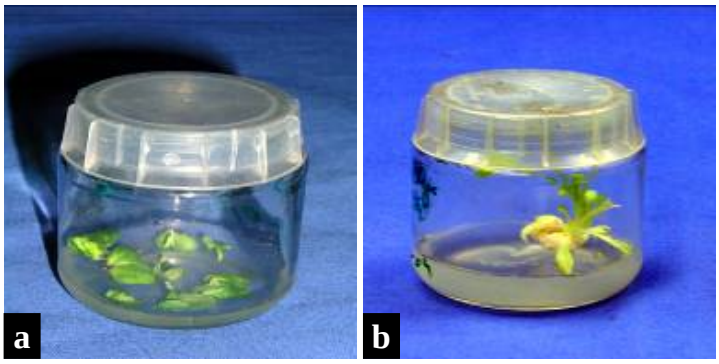

Fig. 2. Callus induction of Cotyledon Culture: (a) Cotyledon cultured on MS-solidified medium, (b) Plantlet regeneration from cotyledon.

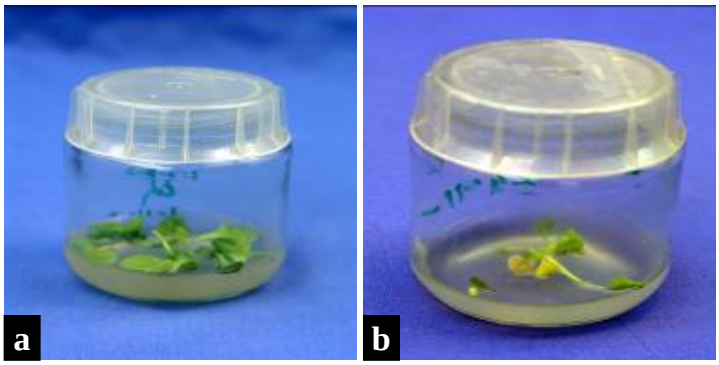

Fig. 3. Callus induction of Leaflet Culture: (a) Leaflet cultured on MS-solidified medium, (b) Plantlet regeneration from leaflet.

Results

Four F_{4-5} populations of *Brassica napus* (*Bn*) crossed with *Brassica campestris* (*Bc*) were investigated for *In- vitro* response for callus induction frequency and interval. A high percentage of explants induced callus. All the explants showed an initial swelling before callus induction.

Seed explants: Callus induction frequency (CIF) for seed explants exhibited a mean percentage of 82.23% (Table 2). Fig. 1 and Table 2 show that mean CIF of seed explants from F_{4-5} population was higher than both *Bn* (81.46 %) and *Bc* (74.06 %) species. Mean CIF of Maluko × 2163 was the highest (88.90 %) among all four populations. Correspondingly, both parental lines of the same population i.e. Maluku (*Bn*, 94.40 %) and 2163 (*Bc*, 77.80%) were the best among parental lines (Table 2).

Generally, all the F_{4-5} populations induced callus earlier than their respective parental lines except Maluko × 2065. This population took 54.67 days in comparison to its parental lines Maluko (51.63 d) and 2065 (57.67 d). Callus induction in Maluko × 2163 population was the earliest (34.44 d) as were their parental genotypes Maluko and 2163 which took 51 and 45.22 days, respectively (Table 3).

Cotyledonary explants: In general, a high percentage (86.97) of cotyledonary explants induced callus (Fig. 1) showing the highest CIF. CIF of F_{4-5} populations were better than their *Bc* parental lines (75.73). Population (A-20-28 × 1203) and their parental lines A-20-28 and 1203 induced the highest (97.9%, 99% and 80.33, respectively) callus in cotyledonary explants (Table 2). It was also observed that callus induction in cotyledonary explants was more rapid than seed explants of F_{4-5} population and took only 12.13 days as interval on callus inducing medium. Similarly F_{4-5} progenies also showed an improvement in callus induction interval (CII) in comparison to their parental cultivars (*Bn*, 12.48 days and *Bc*, 14 days) cultured on similar medium (Table 3). CII in Maluko and its combination with 2163 (Maluko × 2163) was the highest (15.56 d) as compared to parental line 2163 (14.33 d). CII of *Bc* line 1203 was also 14.33 d. The combination of Dunkled × 1203 in F_{4-5} took 10.67 days for callus induction, as compared to their parental lines Dunkled (11.3 days) and 1203 (10 d) (Table 3).

Leaflet explants: Leaflet explants started from callus proliferation from petiole region or cut ends. Fig. 1b depicts different stages of callus induction in leaflet explants. CIF of leaflet explants was about 79.06%, which was less than *Bn* (84.87%) and more than *Bc* (78.73%) species. CIF of leaflet explants in F_{4-5} progenies of Maluko × 2163 was the highest (88%) as compared to other three progenies. Table 2 shows that CIF of Dunkled × 1203 and one *Bc* parental line (2163) was the same (77.80%). On average, CII for leaflet explants of F_{4-5} populations was improved (13.92d) as compared to their parental lines (*Bn*, 14.41d and *Bc*, 15.78d) cultured on similar medium. CII in leaflet explants of Dunkled x 1203 was the highest based on mean performance (13d) as compared to the average

performance of its respective parental lines Dunkled (14.56d) and 1203 (14.33d). The performance of 2 *Bc* lines 1203 and 2163 was the same (Table 3).

Discussion

The response of seed explants to MS medium showed that the most of explants induced callus. The findings are relative to work by Inomata (1985). All the interspecifically hybridized F_{4-5} populations showed high CIF as compared to their parental lines. But in general, a high percentage of explants formed callus. These findings are consistent with Turgut *et al*., (1998). Callus induction for seed explants are always delayed, probably due to the hard seed coat of *brassica*, however, callus was induced about 8 to 9 days earlier in F_{4-5} population as compared to both parental genotypes. Parental lines of *Bn* were a bit late as compared to *Bc* except one line (2065).

Cotyledonary explants responded better than seed and leaflet explants. Muhammad *et al*., (2002) came to similar results during their studies. The findings of the present project support the idea that callus induction is rapid in explants from mitotically active regions of the plant. Coteledonary explants of *Bn* gave high induction frequency as compared to *Bc* parental lines, because of its late maturing nature. Cotyledonary explants of F_{4-5} lines exhibited an initial swelling followed by callus formation within first two weeks of culturing and were earlier than both species. Moreover *Bn* parental lines also took less days as compared to *Bc* parental lines. It was also seen that callus proliferation started from cut ends of the cotyledons.

The frequency of leaflet explants to induce callus on culture medium was lower as compared to seed and cotyledonary explants in F_{4-5} populations, however this was high in *Bn* parental lines. On average F_{4-5} populations took less days as compared to the parental species. In parental species *Bn* was early than *Bc* in callus induction. Leaflet explants with high callus induction interval also exhibited greater quantity of callus in comparison to the ones with low callus induction interval. Most of the leaflet explants managed to induce callus within 2 to 3 weeks of culture. Muhammad *et al*., (2002) came to similar result during their studies.

The *In vitro* culture protocol of the present study was efficiently used to analyze the comparative improvement among parental material and interspecifically hybridized lines in final stages of varietal evolution. Based on the data of the present study we can conclude that the reported system is repeatable and can be easily used to regenerate transgenic canola plants. Using this method we can achieve several primary goals, like the production of canola plants with shorter life span or early maturing canola varieties under *In vitro* conditions. Other secondary goals may be the production of canola cultivars failing to tolerate severe field conditions. *In vitro* production of incompatible *Brassica* genotypes failing to germinate in rigorous vivo environment could also be achieved following the given protocol.

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