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Combining Ability of Callus Induction and Plant Regeneration in Canola (*Brassica napus* L.) Anther Culture

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ABSTRACT

Anther culture response of six genotypes of canola and their 15 F_1 crosses using a half diallel set of crosses was investigated. Results indicated that considerable genetic variation among tested genotypes was observed. The mean values of parents ranged from 1.16 regenerated plants /100 anthers for the Line 28/09 to 3.16 regenerated plants /100 anthers for the Line 5/09.The highest frequencies of shoot and root regeneration were achieved from the three crosses; 5/09 × Pactol, Serw-4 × Serw-8 and 28/09 × Serw-4, while the lowest ones were obtained in the cross Serw-8 × Pactol. Significant and positive heterotic effects were observed in three crosses for all studied traits. Variances due to both general (GCA) and specific (SCA) combining abilities were highly significant for all studied traits. However, the ratio of GCA/SCA mean squares was more than unity for all studied traits, indicating the predominant role of additive gene action in the inheritance of all studied traits. The promising general combiners for all studied traits were Line 5/09 and Pactol as they attained highly significant and positive general combining ability effects. Significant and positive SCA effects were showed in three crosses; 5/09 x Pactol, 28/09xPactol and Serw-4 × Pactol for all studied traits.

Key words: *Brassica napus* L., Anther culture, General and specific combining ability.

Introduction

Oilseed rape (*Brassica napus* L.) is the third, after palm and soybean, most important source of vegetable oil in the world; it contributes significantly to the economy of many countries. Due to its economic value and high performance in an *in vitro* culture, oilseed rape is considered as one of the most suitable species that can be improved through biotechnological techniques. One of the biotechnological methods most useful in basic researches and plant breeding is androgenesis *in vitro* that aims at the development of haploids and doubled haploids (DH). Doubled haploid method, homozygous plants produced in one generation show a homozygosity of 100% compared to the conventional method, which results in an average level of homozygosity of 96.9%, that is, after five to six generations of selfing (Briggs and Knowles, 1967). One of the principal advantages of haploid techniques is the fixation of segregating genotypes occurring at a lower frequency, in which the recessive gene coding for specific traits is combined in the homozygous condition (Friedt and Zarhloul, 2005). This method is highly preferred compared to classical breeding methods. Therefore, microspore culture is the method of choice in plant genetic research and breeding programs.

The first successes with the production of microspore derived embryos from *Brassica* anther cultures were reported by Keller *et al*. (1975) and Thomas and Wenzel (1975). These techniques have been gradually developed and constantly improved (Forster and Thomas, 2005 and Murovec and Bohanec, 2012). A number of factors influence microspore embryogenesis including genotype (Mathias 1988), donor plant genotype, growth conditions, the stage of explant development (Niu *et al*., 1998), pretreatment composition of the culture medium (Chuong and Beversdorf 1985 and Gland-Zwerger, 1995), and environmental conditions during the culture or the diploidization process. The key for increased regeneration efficiency during androgenesis largely depends on the control of two main developmental switches: the induction of microspore cell division and its ultimate commitment to the embryogenic pathway and diploidization rate (Maraschin *et al*., 2005). Most plants obtained from the microspore culture of *B. napus* are haploid, which, in turn, must be diploidized.

The present study attempts to investigate the genetic background of the anther culture of rapeseed plants by using a classical genetic analysis. Therefore, one of the proposed approaches was to use anther

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culture only with responsive genotypes (Andersen *et al*., 1988). On the other hand, the responsiveness of parental genotype to anther culturing affects the responsiveness of hybrid combinations involving them (Zamani *et al*., 2003). Moreover, there is evidence that F_1 hybrids have a higher androgenetic capacity than the parental forms. The genetic research and breeding programs depend on the proper diagnosis of the conditions of quantitative trait inheritance. During the selection process, the information about the combining ability of parental components used for crossbreeding is very important. This knowledge is essential for proper selection of suitable parents in identifying promising hybrids. A common method used in a classical genetic analysis is diallel crossing applied to evaluate the combining ability of parents and progeny generations. One possible way to analyze diallel crosses is the method proposed by Griffing (1956), which divides the total genetic variance for the GCA of parents and the SCA of obtained hybrids. The GCA determines the ability of the tested line of providing an abundant offspring when crossing it with many other lines, while the SCA is characterized by the ability of two different lines of giving an abundant offspring after their mutual crossbreeding (Sprague and Tatum, 1942). The assessment of the general and specific combining abilities allows determining the additive and non-additive types of gene action as well (Falconer, 1967). Thus, the knowledge of combining abilities helps to understand the nature of the action of genes involved in the expression of quantitative traits and predicts the value of further generations (Machikowa *et al*., 2011).

In the literature, there is limited information about the genetics and heterosis in Canola (Etedali *et al.,* 2011). The objectives of the study were (1) to study anther culture response of some canola parental genotypes and their F_1 hybrids (2) to estimate heterosis and combining ability regarding callus induction and plant regeneration from anther culture.

Materials and Methods

The present investigation was carried out at the Cell and Tissue Culture Laboratory as well as the Experimental Farm of the Agronomy Department, Faculty of Agriculture, Al-Azhar University, Nasr city, Cairo, Egypt, during the period from 2013 to 2015.

Six parental genotypes of canola (*Brassica napus* L.) namely; Line 5/09,Line 28/09, Serw -6, Serw -4, Serw -8 and Pactol representing a wide range of diversity for several traits were selected for this study. These canola genotypes were grown and crossed in a diallel mating design, excluding reciprocals at the Experimental Farm in 2013/14 season. The six parental genotypes and their 15 F_1 hybrids were sown at the Experimental Farm in 2014/15 season to obtain the needed anthers.

Flower buds at 3- 5 mm in length were collected when most microspores were at the mid- to late uninucleate stage of development, as assessed by acetocarmine staining of selected squashed anthers. Raceme with flowers at this stage were cut at the base of the branch and tagged. Then, they were put in water and maintained for 5 days at 4 °C in the dark. After cold pretreatment, the buds were surface sterilized with 20% chlorax solution for 7 min. and rinsed 3- 4 times in sterile water. Anthers were aseptically dissected out and cultures in jars containing the N6 induction medium of anther culture (Chu 1978), supplemented with 5 mg/l 2,4-D, 1 mg/l kinetin, 130 gm /l sucrose and 7 gm/l agar. These jars were incubated first for 5-6 weeks in darkness at 28 ˚C.

When the Embryoids / callus induced from the anthers reached the cotyledonary stage, they were transferred to jars containing MS regeneration medium (Murashige and Skoog 1962) supplemented with 3mg/l BAP, 0.3mg/l NAA and 30 g/l sucrose. These jars were incubated for 5- 6 weeks at 25-27°C with 16 h light. Well developed shoots were transferred to 1/2 MS basal medium supplemented with 0.2 NAAmg/l for root initiation, elongation and their development. The number of shoot and root regeneration were counted. Plantlets with adequate root formation were transplanted to small pots with mixture of soil, sand and compost, under plastic cover for three weeks in a growth chamber maintained at 18 ºC and 16 h light per day.

Completely randomized design was applied in this experiment with 21 genotypes and 10 replicates. Each replicate contained 30 anthers, which were placed in jar. The data recorded on all studied traits (callus induction, shoot regeneration and regenerated plants) were subjected to analysis of variance (Steel and Torrie, 1980) to determine the significant differences among genotypes. Heterotic effects were calculated as the deviation of F_1mean from the mid-parent and expressed in percentage. Estimates of combining ability effects were calculated according to the method 2, model 1 of Griffing (1956).

Results and Discussion

Anther culture response of canola parental genotypes and their F_1 hybrids:

Success in obtaining haploid embryos through *in vitro* culture critically depends on the stage of development of microspores in canola. It is known that the microspores of the late unicellular stage development of the genus Brassica can switch from gametophytic to sporophytic pathway (Kott *et al.,* 1988). The successful application of androgensis in canola breeding programs depends on the good androgenic response of genotypes and the high frequency of plant regeneration (Zhang *et al*., 2012). The 15 F_1 crosses and their parents were evaluated for response to anther culturing and capability of regenerated plants. Mean squares due to genotypes, parents and crosses were highly significant for all studied traits (Table 1), revealing the presence of genetic diversity in the material used for all studied traits.

Table 1: Analysis of variance for anther culture traits of six parents and their 15 crosses of canola.

Sources of variation	d.f	Callus induction (%)	Shoot regeneration (%)	Regenerated plants (%)
Genotypes (G)	20	53.68**	1.924**	0.922**
Parents (P)	5	19.09**	2.068**	1.273**
Crosses (C)	14	67.67**	1.984**	0.813**
P *vs* C	1	30.85**	0.362**	0.694**
GCA	5	183.814**	5.409**	2.148**
SCA	15	97.808**	3.899**	2.017**
Error	189	7.28	0.216	0.207
GCA/SCA		1.879	1.388	1.065

**and ** Significant and highly significant at 0.05 and 0.01 probability levels, respectively.*

The response of the anther of six canola parents and their crosses studied are presented in Table (2). Callus was obtained from all canola genotypes tested. The percentage of anthers that developed calli of parents ranged from 43.33% (Serw-4) to 59.33 % (Line 28/09).The two parents; Line 28/09 and Pactol exceeded other parental genotypes in terms of responding anthers. The crosses ranged from 30.67 calli / 100 anthers for the cross Serw-4 × Pactol to 65.33 calli / 100 anthers for the cross 28/09 × Serw-8 (Table 2). The highest response to callus induction frequencies were observed in four crosses (28/09 × Serw-8, 5/09 × Pactol, 28/09 × Serw-4 and 28/09 × Pactol).The present findings are in line with Mathias (1988) and Zhang *et al*. (2012), who stated that the callus induction greatly influenced by genotype.

Table 2: Anther culture response of 15 F_1 crosses and their respective parents in canola.

Genotypes	Callus induction (%)	Shoot regeneration (%)	Regenerated plants (%)
Line 5/09	55.33	4.33	3.16
Line 28/09	59.33	2.00	1.16
Serw-6	51.33	1.63	1.50
Serw-4	43.33	3.50	2.31
Serw-8	46.67	2.50	1.31
Pactol	58.67	3.83	2.83
5/09 × 28/09	45.33	2.00	1.33
5/09 × Serw-6	46.00	4.00	2.30
5/09 × Serw-4	48.67	2.66	1.33
5/09 × Serw-8	40.67	3.00	1.00
5/09 × Pactol	63.33	5.00	3.33
28/09 × Serw-6	56.67	2.33	1.66
28/09 × Serw-4	63.33	2.66	2.33
28/09 × Serw-8	65.33	2.33	1.33
28/09 × Pactol	63.33	3.33	2.00
Serw-6 × Serw-4	33.33	2.00	1.33
Serw-6 × Serw-8	32.00	3.00	2.00
Serw-6 × Pactol	53.33	1.66	1.33
Serw-4 × Serw-8	49.33	4.33	2.66
Serw-4 × Pactol	30.67	1.66	1.33
Serw-8× Pactol	35.33	1.33	1.00
L.S.D.0.05 0.01	2.57 3.37	0.57 0.76	0.56 0.74

The callus derived from anthers was subcultured on MS medium. When the calli were placed onto MS medium, some of calli differentiated into embryoids (Fig. 1). However, some calli did not differentiate and some of calli differentiated into shoots. The mean number of shoot regeneration per 100 cultured anthers ranged from 1.33 to 5.00 (Table 2). The highest frequencies of regenerated shoots were achieved from the three crosses; 5/09 × Pactol, Serw-4 × Serw-8 and 5/09 × Serw-6, while the lowest ones were obtained in the cross Serw-8 × Pactol (Table 2 and Fig. 1). Four crosses appeared to have significantly higher shoot regeneration than the better parents. These results indicated that frequency of anthers capable of producing shoots was affected by genotypes. Similar results were obtained by Sato *et al.* (1989) and Zhang *et al*. (2006).

Fig. 1: Anther culture of canola. A-B) Formation of callus in the cultured anthers. C-D) Shoot regeneration from cultured anthers. E-F) Root initiation from shoot regeneration. G) Regenerated plantlet transplanted from the jar to small pot with mixture of soil, sand and compost in greenhouse.

The regenerated shoots derived from callus when grew 2-3 cm in length. They again cultured on vials with freshly prepared root induction medium to induce root. The values of parents ranged from 1.16 regenerated plants /100 anthers for the Line 28/09 to 3.16 regenerated plants /100 anthers for the Line 5/09 (Table 2 and Fig. 1). Among the crosses, the rooting response of regenerated shoots performances were found better in the cross 5/09 × Pactol (3.33%) followed by the cross Serw-4 × Serw-8 (2.66%) and the cross 28/09 × Serw-4 (2.33%) (Table 2). These results indicated that frequency of anthers capable of producing whole plantlets was affected by genotypes. Similar results were obtained by Alam *et al*. (2009), who found genotypic effect on regeneration plant. The results of the present study are in accordance with previous reports of Guo and Pulli (1996) and Deepak *et al*. (2008), who indicated that the genotype played an important role in anther culture. Moreover, the three crosses (5/09×Pactol, Serw-4×Serw-8 and 28/09×Serw-4) with the highest response in anther culture had parents that exhibited very good as intermediate response (Table 2). These results indicated that one high responding parent could be used to generate responding F_1 crosses, although there is no guarantee of a high response in the crosses because the inheritance of an anther culture response may be more complicated (Masojc *et al*., 1993).

In addition, the data obtained from this study indicate that hybrids originating from one parent with very good or intermediate performance in anther culture would be of value for developing an *in vitro* system with a high production of regenerated plants. El-Hennawy *et al*. (2011) also reached the same conclusion.

The analysis of variance showed highly significant mean squares for parents *vs*. crosses (Table1), indicating that heterotic effects were pronounced for all traits studied.

Estimates of mid-parent heterosis for callus induction, shoot regeneration and regenerated plants are presented in Table (3). Five out of fifteen crosses showed highly significant and positive heterotic effects for callus induction. The highest heterosis values were observed in the two crosses; 28/09 × Serw-4 (23.37%) and 28/09 × Serw-8 (23.26%). Significant and negative mid-parent heterosis was detected in seven cases. Haggag and El-Hennawy (1992) and Etedali *et al*. (2011) also observed significant heterosis for callus induction. Regarding shoot regeneration, seven crosses exhibited a significant increase of heterosis in this trait than the mid-parent ranging from 3.55 to 45.28%. Negative and significant mid-parent heterosis was observed in eight crosses (Table 3). Ono and Takahata (2000) reported heterotic effect for shoot regeneration. Estimates of mid-parent heterosis for regenerated plants are presented in Table (3). Six out of fifteen crosses showed highly significant and positive heterosis values for this trait. However, negative heterotic effect was observed in eight crosses. El-Hennawy *et al*. (2011) reported also heterotic effect for pollen callus induction and plant regeneration.

Table 3: Heterosis as percentage of mid parent for anther culture traits of canola.

Genotypes	Callus induction (%)	Shoot regeneration (%)	Regenerated plants (%)
5/09 × 28/09	-20.93**	- 36.80**	- 38.42**
5/09 × Serw-6	-13.75**	34.22**	-1.28**
5/09 × Serw-4	-1.35	- 32.09**	- 51.91**
5/09 × Serw-8	-20.26**	- 12.17**	- 55.38**
5/09 × Pactol	11.11**	22.54**	11.18**
28/09 × Serw-6	-1.15	28.45**	24.81**
28/09 × Serw-4	23.37**	- 3.27**	34.29**
28/09 × Serw-8	23.26**	3.55**	7.69**
28/09 × Pactol	7.34**	14.24**	0.25
Serw-6 × Serw-4	-29.57**	- 22.03**	- 30.18**
Serw-6 × Serw-8	-34.69**	45.28**	42.34**
Serw-6 × Pactol	-3.03	- 39.19**	- 38.56**
Serw-4 × Serw-8	9.62**	44.33**	46.96**
Serw-4 × Pactol	-39.86**	- 54.78**	- 48.24**
Serw-8× Pactol	-32.92**	- 57.97**	- 51.69**

**and ** denote significant at 0.05 and 0.01 levels of probability, respectively.*

General and specific combining ability effects *in vitro* of canola:

Information on combining ability of plant regeneration in anther culture is of great importance in an attempt to increase the efficiency of anther culture. Zamani*et al.* (2003) reported that by understanding the inheritance patterns of anther culture response, breeders could improve the procedures by crossing highly responsive with non-responsive genotypes, and could predict the level of response of the hybrids on optimize the allocation of resources for doubled haploid production.

Results indicated that there were highly significant differences due to both general and specific combining abilities (Table 1). Therefore, it seemed that both additive and non-additive types of gene action were operative for all studied traits. However, the ratio of GCA/SCA was more than unity for all studied traits, indicating the predominant role of additive gene effects in the expression of all studied traits. Similar results were reported by Ghaemi *et al*. (1995), Ono and Takahata (2000) and Dagüstü (2008). On the other hand, Hansen *et al*. (1999), Al-Ashkar (2014) and Turczynowska *et al.* (2015), found that general combining ability estimates were less than specific combining ability for callus induction and plant regeneration.

Estimates of GCA effects for each parent are presented in Table (4). Results revealed that general combining ability effects ranged from - 4.388 to 8.194 for callus induction, from -0.847 to 1.402 for shoot regeneration and from -0.555 to 0.694 for regenerated plants. The three parental genotypes; Line 28/09, Pactol and Line 5/09 showed highly significant and positive general combining ability effects for callus induction, whereas the remaining three varieties gave poor general combining ability effects for this trait. The parents; Line 5/09, Serw-4 and Pactol were considered to be the best general combiners for shoot regeneration and regenerated plants as they showed highly significant and positive GCA effects for these traits. While, the other three genotypes were poor combiners as they showed highly significant and negative general combining ability effects for these traits.

It is interesting to note that the parents; Line 5/09 and Pactol proved to be the best general combiners for all studied traits. Therefore, both parents could be used in the future breeding programs for genetic improvement of the callus induction and plant regeneration in rapeseed. On the other hand, significant and negative general combining ability effects were observed in two parental genotypes (Serw-6 and Serw-8)

for all studied traits.

Estimates of specific combining ability effects for the studied crosses are given in Table (4). Significant positive SCA effects were recorded in seven crosses for callus induction trait representing three

Table 4: Estimates of general combining ability (GCA) and specific combining ability (SCA) effects for the studied traits of canola.

Genotypes	Callus induction (%)	Shoot regeneration (%)	Regenerated plants (%)
Line 5/09	0.944**	1.402**	0.694**
Line 28/09	8.194**	-0.763**	-0.472**
Serw-6	- 4.388**	-0.847**	-0.305**
Serw-4	- 3.888**	0.152*	0.194**
Serw-8	- 2.888**	-0.180*	-0.555**
Pactol	2.026**	0.236**	0.444**
SE(gi)	0.424	0.061	0.060
SE (gi-gi)	0.657	0.095	0.093
		F_1's	
5/09 × 28/09	- 13.392**	-2.274**	-1.238**
5/09 × Serw-6	- 1.642	1.809**	0.595**
5/09 × Serw-4	2.523*	-1.857**	-1.905**
5/09 × Serw-8	- 5.976**	-0.857**	-1.821**
5/09 × Pactol	10.773**	2.726**	1.845**
28/09 × Serw-6	1.773	0.643**	0.428*
28/09 × Serw-4	9.940**	0.309	1.261**
28/09 × Serw-8	11.440**	-0.024	0.011
28/09 × Pactol	3.523**	1.559**	0.345*
Serw-6 × Serw-4	- 8.976**	-0.940**	-0.905**
Serw-6 × Serw-8	- 12.392**	-3.024**	-1.571**
Serw-6 × Pactol	-10.809**	1.393**	1.178**
Serw-4 × Serw-8	4.607**	-1.690**	-1.155**
Serw-4 × Pactol	8.023**	3.059**	2.012**
Serw-8× Pactol	-16.559**	-2.690**	-1.655**
SE(sij)	1.164	0.169	0.165
SE (sij-sik)	1.738	0.252	0.246

**and ** Significant and highly significant at 0.05 and 0.01 probability levels, respectively.*

types of combinations;good × good, good × poor and poor × poor general combiners. The highest SCA effect for this trait was observed in two crosses(28/09 × Serw-8 and 5/09 × Pactol), including two types of combinations; good × poor and good × good general combiners. On the contrary, significant negative specific combining ability effects were observed in six crosses. Regarding shoot regeneration, six crosses exhibited positive and significant SCA effects including good × good, good x poor and poor x poor general combiners. Therefore, these crosses could be of great value for varietal improvement programs. On the contrary, significant negative specific combining ability effects were observed in seven crosses. Concerning regenerated plants, seven crosses showed significant and positive SCA values including good × good, good × poor and poor × poor general combiners for this studied trait. On the other hand, significant negative specific combining ability effects were observed in seven crosses. However, it is not necessary that parents having high estimates of GCA effects would also give high estimates of SCA effects. For instance, the cross (5/09 × Serw-4) involving parents with high general combining ability effects for number of regenerated plants / 100 cultured anthers gave very low specific combining ability effects. Gill *et al*. (1972) stated that the low SCA effects in such cases might be attributed to some internal cancellation of favorable factors or to genetic similarity of the involved parents. In contrast, the cross (28/09 × Serw-6) involving parents with very low general combining ability effects for number of regenerated plants / 100 cultured anthers gave high specific combining ability effects for this trait, which might be due to high genetic diversity among the parents. Moreover, the parents having low GCA effects had a relatively high magnitude of non-additive gene effects and thus resulted in high SCA effects when crossed.

It is worthy to note that the highest specific combinations for all studied traits were presented in three crosses (Serw-4 × Pactol, 5/09 × Pactol and 28/09 × Pactol) including two types of combinations good × good and good x poor general combiners. Therefore, these crosses could be of great value for anther culture selection system in a canola improvement program.

In conclusion, the existing genetic variation and predominance of additive gene action in genetic control of callus induction and plant regeneration traits indicated that plant regeneration ability in anther

culture can be improved through crossing and selection in canola breeding programs.

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