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Development of Haploids and Double Haploids in Oil Seed Crop through Anther Culture

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Abstract: ***Anther Culture or microspore culture is a very important and useful tool in plant breeding for efficient production of haploids and subsequent doubled haploid plants in many species, limited research has been conducted with Brassica –rapa as it is more recalcitrant in cell and tissue culture than other Brassicas. In order to access the effect of genotype on microspore embryogenesis in B – rapa, two high yielding brown Sarsoon cultivars (KS-101 and KOS-1) were studied for their response to haploid plant regeneration through Anther culture. Flower buds of 2.2 mm to 2.7 mm size representing late uninucleate stage of anther microspores were subjected to low temperature treatment 4^0C for 7days and anthers from these buds were cultured on MS medium having 13% sucrose. Anthers were then sub cultured in MS media supplemented with various concentrations of auxins and cytokinins. The best callus development and proliferation was achieved in MS media supplemented with 2,4-D 1mg/l and 0.5 mg/l NAA. Sucrose concentration, cold pre treatment and incubation time influenced embryogenesis. For regeneration of haploid plantlets anther derived calli were transferred to MS full strength medium supplemented with kn92.0mg/l) fallowed by the use of BA (2.0mg/l) and incubated at 22 ± 20C in light and gave maximum regeneration of 75%. The regeneration shoots (at three leaf stage with length of 5cm) were subculture in rooting media and maximum rooting (30.07%) was achieved when MS medium was supplemented with IBA (0.4mg/l). Root tip mitosis chromosome counts revealed percentage of haploid frequency of KOS-1(41.85) followed by KS-101(38.84%).After 5 days of rooting root samples were taken for confirmation of haploid nature of the plant, Haploid frequency was greater than 65%.***

Keywords: Haploids, *Brassica-rapa*, Shoot/ Root regeneration

1. Introduction

Brown sarson is the major oilseed crop of valley grown on about 50 thousand hacters and fits in rotation with Rice crop. No significant breakthrough in enhancing the existing productivity levels (7-8 q/ha) and oil quality has been possible due to lack of variability in the available germplasm (Singh *et al* 2007) . Operation of self incompatibility limits production of homozygous inbred lines through conventional breeding procedures, necessitating biotechnological interventions for accelerating breeding progress and generating genetic variability useful for developing high yielding varieties.The efficient production of haploid and double haploid (DH) plants from anther or microspore culture has become an important new tool for *Brassica* breeders. But limited research has been conducted with Brown Sarson(*Brassica rapa*) as it is more recalcitrant in cell and tissue culture than other *Brassicas*. The present investigation was undertaken to develop a protocol for production of DH Plants in *B.rapa* from anderogenic embryos.

2. Material and Methods

Flowered buds 2.5 to 2.7 mm long representing late uninucleate stage were collected from field grown plants of 2Brassica compestris varieties namely KOS-1 and KS-101 , subjected to low temperature pretreatment and surface sterilized with 1% sodium hypochloride for 8 to 10 minuts (Gu et al 2004). Anthers were picked from buds and cultured on MS medium suplimented with various concentrations of 2,4-D and NAA for embryogenic callus induction.For regenerations from embryogenic callus MS media suplimented with various concentrations of auxins and cytokinens were used. Root induction in regenerated shoots was tried using various concentrations of IBA. The cultures were incubated at 22+- 2^0 C. Root tip mitotic chromosome counts were used to confirm haploid nature of Anther derived regenerates and double haploidy level induced through use of various concentrations of colchicines was detected cytologically using pollen mother cell (PMC).

3. Results and Discussion

Maximum percentage of aseptic culture was achieved in KS-101followed by KOS-1genotypes.Callus initiation was maximum (32.62) when anthers were given pretreatment (chilling treatment) temperature of 4^0C for seven days (Zhang *et al* 2006,Kumari and Singh 2014) .Maximum percentage of callus initiation (22.48) was observed in KS-101 genotype followed by KOS-1(Gu etal 2003)[Table 1].

Maximum number of anthers per 100 buds forming callus was observed in Murashige and Skoog medium 1962, supplemented with 2,4-D (1.0 mg/lit) and NAA(0.5 mg/lit) (36.60). Days taken to initiate callusing was minimum (22.48 days) when induction medium (MS medium) was supplemented with BA (1.5 mg/lit) and 2,4-D (1.0 mg/lit)(Li etal .,2005)

Highest number of shoots per explants of proliferated cultures (86.00) was achieved (Natalaji *etal* 2006) [Table 2] when MS full strength medium was supplemented with Kn (2.0 mg/lit) followed by the use of BA(2.0 mg/lit)(69.00), highest number of shoots was regenerated in KS-101(53.83)followed by KOS-1(53.66).These shoots devoid of any callus, were sub-cultured on rooting medium containing various concentrations of auxin (IBA). Maximum

rooting (30.75%) was achieved when MS medium was supplemented with 0.4mg/lit IBA[Table 3]. Root tip mitosis chromosome counts revealed maximum percentage of haploid frequency in KOS-1(41.85%) followed by KS-101(38.84%)(Natalija *etal* 2004)[Table 4]. Diploidization of haploid plants was carried out by using different concentrations of colchicines for different time durations (Dwarkesh *etal* 2006).Maximum doubling efficiency was obtained when roots of plantlets were submerged in 20mg/lit colchicines for 2 hours and about 40-45 per cent diploidization was achieved.

Table 1: Effect of pre treatment of anthers on callus initiation

Genotype	T1	T2	T3	T4	T5	T6	Mean
KOS-I	23.86 (29.22)	21.88 (27.87)	22.88 (28.55)	30.25 (33.35)	23.02 (28.65)	21.01 (27.26)	**23.81 (29.15)**
KS-IOI	22.03 (27.97)	20.98 (27.24)	21.98 (27.94)	45.78 (42.57)	23.34 (28.88)	19.23 (25.99)	**25.55 (30.10)**

Main effect LSD ≤ (p = 0.01) S.E T_1=1^{0}C
Genotype (GT) = 1.44 0.60 T_2 = 2^{O}c
Temperature (T) = 1.10 0.49 T_3 = 3^{0}c
Interaction effect T_4 = $_4{}^0$c
GT x T = 3.12 1.20 T_4= $_5{}^0$c T_6 = $_6{}^0$

Growth regulatormg/l	KS-101	KOS-1
BA (0.0)	32.00	48.00
BA(2.0)	69.00	63.00
BA(3.0)	48.00	46.00
Kn(0.0)	44.00	42.00
Kn(2.0)	86.00	72.00
Kn(3.0)	44.00	51.00
Mean	53.83	53.66

Table 2: Influence of growth regulators on number of plants regenerated

Effects		C.D	S.E
Main effect			
Genotype	(GT)	0.77	0.36
Growth regulators (GR)	**1.1**	**0.47**	

Table 3: Influence of growth regulators on per cent *in vitro* rooting of micro shoots

Growth Regulators	KOS-1	KS-101
IBA(0.0)	24.01(29.33)	18.06(25.14)
IBA(0.2)	25.14(30.09)	20.09(26.62)
IBA(0.4)	26.16(30.75)	23.08(28.71)
IBA(0.6)	21.11(27.35)	19.39(26.12)
IBA(0.8)	19.20(25.98)	18.66(25.58)
Mean	**23.12(28.70)**	**19.89(26.44)**

LSD (p ≤ 0.01) S.E ±

Main effect
Growth regulator (GR) = 0.78 0.32
Genotype (GT) = 0.88 0.36

Interaction effect
GR x GT = 1.95 0.72

Table 4: Influence of different treatment combinations and media formulations on haploid frequency (Per cent)

Growth regulators mg l-1	Number of plants regenerated	
	KOS-1	KS-101
BA (0.0)	24.04 (29.36)	18.44 (25.42)
BA (2.0)	30.22 (50.89)	58.25 (49.74)
BS (3.0)	40.21 (39.35)	37.25 (37.61)
Kn (0.0)	30.24 (33.36)	25.24 (30.15)
Kn (2.0)	56.22 (48.57)	54.22 (47.42)
Kn (3.0)	40.22 (39.35)	39.66 (39.03)
Mean	41.85 (40.15)	38.84 (38.23)

Effect LSD ≤ (p = 0.01) S.E ±
Main effects
Growth regulator (GR) = 0.25 0.12
Genotype (GT) = 0.20 0.10

Interaction effect
GR x GT = 0.50 0.24
* Data in parenthesis are transformed values (Sin^{-1} √p)

4. Conclusion

Protocol developed for the production of double haploid plants through endrogenesis offers a viable alternative to develop homozygous lines for further use in the development of hybrid/synthetic varieties having higher yield, better quality and tolerance to stresses.

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