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In vitro Regeneration of Plants in *Pisum sativum* and their Reaction to *Ascochyta pinodes*

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The leaf, epicotyl and root explants of *Pisum sativum* cv Arkel were used for establishing callus cultures on MS and B5 media containing various supplements. MS medium supplemented with 2, 4-D, NAA and BA, each at 0.5 mg/l, was found best as all the explants gave 100% callus formation. Plantlet regeneration was obtained on B5 medium supplemented with sucrose (20 g/l), BA (2 mg/l) and NAA (0.5 mg/l). Maximum root induction (93.5%) in tissue culture derived shoots was obtained on half strength B5 medium supplemented with sucrose (20 g/l) and NAA (0.5 mg/l). Rooted plants were easily established in pots in sterilized potting mixture. R1 progenies of eighteen somaclones evaluated for their reaction to *Ascochyta pinodes* showed that somaclones with high degree of resistance to this pathogen could be selected. Five somaclones, A 6-2, A 6-1, A 5-2, A 7-1 and A 10-1 with small spot size and less number of pycnidia/ spot as compared to the parent cultivar Arkel were obtained.

Key Words : *Ascochyta pinodes*, *Pisum sativum*; Somaclonal variation; Tissue culture

Abbreviations

BA, 6-benzylamino purine; *IAA*, Indole acetic acid; *IBA*, Indole-3-butyric acid; *MS*, Murashige and Skoog (1962); *2, 4-D*, 2, 4-dichlorophenoxyacetic acid; *NAA*, Naphthalene acetic acid

Introduction

Grain legumes, in general, are known to be recalcitrant as far as *in vitro* plant regeneration is concerned (Morginski & Kartha 1981). Lack of stable and effective regeneration system in *Pisum sativum* was a major obstacle in the exploitation of this technique for pea improvement until the reports of Kysely et al. (1987) and Kysely and Jacobsen (1990) who reported whole plant regeneration in pea via somatic embryogene- sis. Somatic embryogenesis in *P. sativum* has also been reported by other workers (Ozcan et al., 1993, Loiscau et al. 1995, Doorne et al. 1995). However, root formation in tissue culture derived pea shoots occurs in low

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frequency (Kysely et al., 1987, Naverby et al 1991, Cardi et al., 1991) thus limiting the success of transferring these plants into soil. To our knowledge, there is no report on the selection of somaclones of *P. sativum* for reaction to *Ascochyta pinodes* causing Ascochyta blight of pea. This communication reports the callogenic potential of different explants, high frequency root induction in tissue culture derived shoots and reaction of somaclones of *P. sativum* to *A. pinodes* causing Ascochyta blight.

Table 1 *Callus induction from different explants of* ***Pisum sativum*** *cv Arkel on media containing different supplements*

Medium	Explant source	Explants transferred (no.)	Calli formed (no.)	Callus formation (%)
M1	Epicotyl	11	10	90.9
	Leaf	6	5	83.3
	Root	7	5	71.4
M2	Epicotyl	10	10	100.0
	Leaf	10	10	100.0
	Root	7	6	85.7
M3	Epicotyl	5	5	100.0
	Leaf	-	-	-
	Root	9	8	88.9
M4	Epicotyl	5	5	100.0
	Leaf	10	10	100.0
	Root	5	5	100.0

M1 = B5 +2, 4-D + BA (0.5 mg/l each); M2 = B5 + NAA 2mg/l + BA 1 mg/l; M3 = MS + 2, 4-D and NAA 0.2 mg/l each + Kinetin 2 mg/l; M4=MS + 2, 4-D + NAA + BA (0.5 mg/l each); - not tested

Materials and Methods

Establishment of callus cultures and shoot regeneration : An early maturing *P. sativum* cultivar, Arkel cultivated commercially in Himachal Pradesh (India) was used in the present study. To obtain leaf, epicotyl and root explants, 50 seeds were surface sterilized with 0.1% aqueous solution of $HgCl_2$ for 5 min. followed by three washings with sterilized distilled water and germinated on sterilized half strength MS (Murashige & Skoog 1962) medium in 250 ml Erlenmeyer flasks. Leaf, epicotyl and root explants were obtained from 12 day old seedlings and inoculated on MS and B5 (Gamborg et al. 1968) media, supplemented with 3% sucrose (BDH, AR), 0.8% agar (Bacteriological grade) and four concentrations of 2, 4-D, BA, Kinetin and NAA. The *p*H of media was adjusted to 5.8 before autoclaving at 121°C and 1.06 kg/cm^2 pressure for 20 min. Observations on callus formation were recorded after 30 days of incubation. For shoot regeneration, 5-6 wk old calli were transferred onto B5 medium containing 2% sucrose, 0.8% agar, 2 mg/l BA and 0.5 mg/l NAA. Shoot buds obtained after 4 wk of culture were maintained by subculturing on the same medium for multiple shoot formation and better shoot growth. All experiments were conducted in a growth room at 25 ± 1°C and 16 hr light/8 hr dark cycles of 3500-4000 lux light intensity.

Root induction and acclimatization of in vitro derived plantlets : For root induction, basal media like T (Nitsch & Nitsch 1969), half strength B5 and MS containing 2% sucrose, 0.7% agar and five concentrations of NAA were poured into culture tubes (25 × 150 mm), which were plugged and sterilized as described earlier. Tissue culture derived shoots (approx. 3cm) were separated from calli with the help of sterile blade and placed vertically on the rooting medium.

Plantlets with well developed root systems were removed from the medium. The roots were thoroughly washed under running tap

water. These plantlets were transferred to sterilized potting mixture of sand, soil and farm yard manure (1 : 1 : 1 w/w) in plastic pots (5 cm dia) and maintained for 14 days under highly humid conditions. The humidity was maintained by inverting 250 ml beakers or polythene bags on the pots containing tissue cultured plants. Acclimatized plants were transferred to large plastic pots (25 cm dia) containing unsterilized potting mixture and transferred to green house benches. Seeds were collected from ten tissue cultured plants (R0) upon maturity.

Table 2 *Root induction on different media in tissue culture derived shoots of* ***Pisum sativum*** *cv Arkel*

Medium	Shoots transferred (no.)	Shoots with roots (no.)	Root formation (%)
T medium	20	1	5.0
1/2 B5 + NAA (0.2 mg/l)	12	8	66.6
1/2 MS + sucrose (15 g/l) + NAA (2 mg/l)	10	1	10.0
1/2 B5 + NAA (0.25 mg/l)	5	1	20.0
1/2 B5 + NAA (0.5 mg/l)	31	29	93.5
1/2 B5 + NAA (1.0 mg/l)	8	4	50.0
1/2 B5 + NAA (1.5 mg/l)	7	4	55.5
1/2 B5 + NAA (2.0 mg/l)	6	2	33.3
Check (1/2 B5)	6	1	16.4

T medium, Nitsch and Nisch, 1969; B5, Gamborg et al. 1968; MS, Murashige and Skoog 1962

Evaluation of somaclones for reaction to Ascochyta pinodes : R1 progenies of eighteen somaclones of *P. sativum* cv Arkel were grown in the experimental area of the Department of Plant Pathology, HPKV, Palampur during 1993-94. Two leaves (5th and 6th from the base) from each plant were taken for screening against *A. pinodes* in detached leaf cultures (Sharma 1990). Leaves were kept in the Petri plates lined with moist blotting papers. Pure culture of *A. pinodes* was obtained from the Department of Plant Pathology, HPKV, Palampur. The culture was multiplied on oat meal agar medium for 7 days. Spore suspension was prepared in distilled sterilized water from the actively growing culture with mature pycnidia. A single drop (0.02 ml) of spore suspension of *A. pinodes* (1500-2000 spores/ml) was placed on each half of the leaflets. Observations on spot size and number of pycnidia/spot were taken after twelve days of inoculation. Number of pycnidia/spot were recorded with the help of magnifying lens (10 x). Replication wise means of each genotype were subjected to statistical analysis. Analysis of variance was based on the linear model suggested by Mead et al. (1993) Genotypic and phenotypic co-efficients of variance, and estimates of heritability (broad sense) and genetic advance (% of mean) at 5% selection intensity were estimated following Burton and DeVane (1953).

Results and Discussion

Frequency of callus induction in leaf, epicotyl and root explants of *P. sativum* cv Arkel is given in table 1. All epicotyl explants of cv Arkel were formed into calli on each of the test media except M1 where 90.9% callus induction was obtained. Callus formation from leaf explants was maximum (100%) on M2 and minimum (83.3%) on M1 medium. However, callus induction from root explants was 71.4% and 88.9% on M1 and M3 media, respectively. Epicotyl and leaf explants showed very good response in

terms of callus induction on all the media tested. Earlier studies also reported establishment of callus cultures from leaf, epicotyl and root explants (Jacobsen et al., 1980, Doorne et al. 1995). In the present study MS medium containing 2, 4-D NAA and BA (0.5 mg/l each) was found an excellent callus inducer. Jacobsen et al. (1980) and Loiscau et al. (1995) have also reported that cytokinin alongwith 2, 4-D increased callus growth of pea epicotyl. However, Kunakh et al. (1984) reported substitution of 2, 4-D with IAA or NAA had negative effect on callus induction.

Calli obtained from root and epicotyl explants were compact, creamish white in colour and slow growing on all the test media. However, fast growing green, friable calli with small nodular structures were obtained from leaf explants. Hard whitish calli from root explants and yellowish brown friable calli from epicotyl explants have been reported by Jacobsen et al. (1980). Friable calli with green nodular structures obtained from leaf explants in the present study were due to the formation of somatic embryos. This type of callus obtained from shoot apices and immature embryos has been used for somatic embryogenesis in pea (Kysely et al. 1987, Kysely & Jacobsen 1990).

Calli obtained from different explants were sub-cultured on B5 medium supplemented with sucrose (20 g/l), BA (2 mg/l) and NAA (0.5 mg/l) for plant regeneration. After 3-4 wk of culture, small shoot bud primordia were obtained from calli derived from leaf explants. Shoot bud primordia were separated from the callus and transferred onto the same medium. Numerous shoots were formed within 4 wk of culture. Well developed four to six shoots were obtained after another 4 wk of culture. Regenerability of shoot bud primordia was not reduced with repeated subculturing on the same medium. Only the nodular calli derived from leaf explants regenerated into plantlets. Kysely et al. (1987) and Kysely & Jacobsen (1990) have reported plant regeneration from shoot apices and immature embroys of pea via somatic embryogenesis. Although plant regeneration from calli obtained from epicotyl and root explants was not obtained in the present investigation, Kunakh et al. (1984) were able to regenerate shoots from callus cultures obtained from root, epicotyl and leaf explants.

Tests for root induction in tissue culture derived shoots of *P. sativum* cv Arkel on different media showed that maximum root induction (93.5%) was obtained on half strength B5 medium containing sucrose (20 g/l) and NAA (0.5 mg/l) followed by half strength B5 containing sucrose (20 g/l) and NAA (0.2 mg/l). Root initiation started after 10-12 days of culture on these two media and the plants were ready for transfer to soil after 20 days of incubation. Root formation on two other media at different hormonal levels (NAA) ranged from 5.0-55.5%. Root initiation was very slow on these media and it took 25-30 days for complete root development and subsequent transfer of plants to soil.

It was also observed that shoots with node at the cut end rooted very fast. The number of roots/shoot ranged from 4-6. However, where the node was absent, callus formation took place at the cut end which inhibited root formation on these root inducing media. Fifteen well rooted plantlets transferred to the pots and kept under highly humid conditions for 12-15 days were transferred to large pots (20 cm dia) under green house conditions till plant maturity

and pod formation. All the ten R0 plants were fertile having few (2-3) pods per plant.

Although there are a number of reports on the induction of roots in tissue culture derived shoots of *P. sativum*, a satisfactory and rapid method with high frequency of root induction was not available. Kunakh et al. (1984) were unable to induce roots in the shoots derived from long term cultures of calli of *P. sativum*. Naverby et al. (1991) reported that 'T' medium gave a maximum of 49% rooting frequency in pea cv Puget. But in the present study rooting could be induced only in 5% of the cultured shoots. Cardi et al. (1991) reported that NAA and IBA 0.2 mg/l could induce better *in vitro* root formation in pea cultivars. Another significant observation was that if the tissue culture derived shoots having a node at the cut end are cultured on the rooting medium, these result in high frequency and fast root formation.

Table 3 *Analysis of variance for spot size of* ***Ascochyta pinodes*** *in somaclones (R1) of* ***Pisum sativum*** *cv Arkel*

Source of variation	df	MS	F/cal
Replication	1	1.37	
Genotype	18	85.91**	3.42
Error	18	25.12	

** significant at 1% level

Reaction of somaclones (R1) to Ascochyta pinodes : Somaclonal variation for disease resistance in *P. sativum* has not been reported as yet. However, quantitative and qualitative variations for total proteins and isozymes have been obtained (Mikhailova et al. 1991 and Gria et al. 1995). In the present investigation, analysis of variance of spot size of *A. pinodes* on somaclones of *P.*

Table 4 *Estimates of parameters of variability with respect to spot size of* ***Ascochyta pinodes*** *in somclones (R1) of* ***Pisum sativum*** *cv Arkel*

Parameters of variability	Values
Range (mm^2)	4.45-33.40
Mean	16.78 ± 3.54
Phenotypic variance	65.51
Genotypic variance	30.39
Phenotypic co-efficient of variance (PCV%)	48.22
Genotypic co-efficient of variance (GCV%)	32.85
Heritability (%)	46.39
Genetic advance (percentage of mean)	45.70

Table 5 *Mean performances of somaclones (R1) of* ***Pisum sativum*** *cv Arkel for spot size and number of pycnidia/spot of* ***Ascochyta pinodes***

Soma-clones	Spot size[1] (mm^2)	Pycndia/ spot (no.)	Soma-clones	Spot size (mm^2)	Pycndia/ spot (no.)
Arkel	33.40	11.75	A4-1	14.60	1.42
A1-2	26.57	7.25	A1-1	4.45	4.06
A4-2	25.54	6.50	A9-1	13.36	5.33
A10-2	23.97	3.50	A5-1	12.67	5.68
A2-1	20.71	7.09	A10-1	12.57	4.25
A8-1	18.62	7.83	A7-1	11.34	6.29
A7-2	17.49	8.16	A5-2	10.22	6.17
A8-2	15.08	8.44	A6-1	10.10	4.82
A9-2	15.04	4.22	A6-2	8.45	4.05
A3-2	14.70	7.20			

[1]SE (m) ± 3.54

SE (d) ± 5.01

CD ± 10.53

sativum revealed significant differences among somaclones (table 3). Spot sizeranged from 4.45-33.40 mm^2 with an average of 16.78 ± 3.54. Variation for reaction to *A. pinodes* in the somaclones was highly significant with respect to spot size (table 3). Sufficient variation was observed for the trait as evidenced from high co-efficients of phenotypic (48.23%) and genotypic (32.85%) variation suggesting the scope of selection for smaller spot size in the present material (table 4). Low heritability broad sense (46.39%) coupled with high genetic advance (45.70%) indicates the significance of environmental factors in the expression of this trait. Barring somaclone No A1-2, all the somclones differed from Arkel (table 5). The somaclones with smaller spot size like, A6-2, A6-1, A5-2, A7-1 and A10-1 can further be used in pea breeding programme aimed at developing pea varieties with high degree of resistance to *A. pinodes*.

Data on pycnidium-density/spot are given in table 5. Number of pycnidia/spot varied from 3.5 in somaclone A10-2 to 8.44 in A8-2. Spots on somaclone A10-2 had very few number of pycindia in spite of large spot size. Selection of somaclones with less pycnidium density in Ascochyta spots would be useful in checking the secondary spread of the pathogen.

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