

Generation and characterization of pea (*Pisum sativum*) somaclones for resistance to *Ascochyta* blight and powdery mildew

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Somaclones of two pea cultivars, Palam Priya and Lincoln were generated and evaluated for resistance to *Ascochyta* blight and powdery mildew. Among the hormonal combinations tested for callus induction in MS medium, the best combination observed was 2,4-D + NAA + BAP 0.5 mg/l each using leaf explants. Frequency of callus differentiation was maximum (10 and 23.5% for Palam Priya and Lincoln, respectively) on MS medium containing NAA 0.5 mg/l + BAP 6 mg/l but shoots formed withered and did not root either. However, MS medium containing NAA 0.5 mg/l + BAP 5 mg/l gave good differentiation. *In vitro* flowering as well as seed formation was also observed in 11 shoots. Vigorous rooting was obtained when *in vitro* grown shoots having node at cut end were cultured on half strength MS medium containing NAA 2 mg/l. Five of 71 Lincoln somaclones and 1 of 21 Palam Priya somaclones showed resistance to *Ascochyta pinodes* but none were resistant to *Erysiphe pisi*. However, one somaclone (SP6-1) of Palam Priya possessing resistance to *Ascochyta* blight also showed moderate resistance to powdery mildew. Increased PAL as well as peroxidase activity was observed in somaclones showing resistance to *Ascochyta* blight. Variation in the banding pattern of esterase was also observed in the native-PAGE profiles of Palam Priya and Lincoln, and somaclones derived from them.

Keywords: *Ascochyta pinodes*, *Erysiphe pisi*, *in vitro*, isozymes, pea, *Pisum sativum*, somaclones

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Introduction

Pea is an important *rabi* crop of Himachal Pradesh and is also grown in the dry-temperate regions during off-season (summer) as a cash crop. The crop is vulnerable to a large number of diseases, such as powdery mildew (*Erysiphe pisi*), *Ascochyta* blight (*Ascochyta* spp.), bacterial blight (*Pseudomonas syringae* pv. *syringae*), white rot (*Sclerotinia sclerotiorum*) and pea rust (*Uromyces pisi*). Of these, powdery mildew and *Ascochyta* blight pose a continuous threat to its successful cultivation in the crop growing areas of the state. *Ascochyta pinodes* is the most devastating species in Himachal Pradesh and causes leaf blight and spots on leaves and pods¹. So far, source of resistance to these two pathogens has not been identified among a wide array of pea germplasm. Production of resistant variants, therefore, remains the only and eco-friendly approach for their management.

Plant tissue culture techniques have been used for generating variants, which differ from the parental types, a phenomenon known as somaclonal variation².

Generation of somaclones is one of the most promising areas where cell culture can generate itself stable and heritable variation useful for crop improvement. This variation is thought to derive either from the expression of genetic diversity pre-existing in the explant, or else from variability during cell de-differentiation or callus maintenance phase. Frequency of variants might depend upon the genotype involved, the type of explant, culture media used and cultural conditions. Plant regeneration in pea via somatic embryogenesis has been achieved by various workers^{3,4}. However, rooting in the tissue culture derived shoots has been reported to be very low⁵; thus, limiting the successful transfer of the *in vitro* grown plantlets to the soil and their further evaluation against biotic/abiotic factors.

Keeping in view the need to induce resistance in pea against *Ascochyta* blight and powdery mildew, an attempt was made to generate somaclones from locally grown cultivars Lincoln and Palam Priya. The somaclones thus obtained were evaluated for resistance against *A. pinodes* and *E. pisi*. Resistant variants were also analyzed for phenylalanine ammonia lyase (PAL), esterase and peroxidase isozymes, along with

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the susceptible checks as these enzymes are known biochemical parameters of resistance.

Materials and Methods

Establishment of Callus Cultures

Dry, uniform and bold seeds of pea cultivars, Palam Priya and Lincoln were used for raising seedlings under aseptic conditions. Seeds were surface sterilized with 90% ethyl alcohol followed by a 5 min dip in 0.1% HgCl_2 containing a few drops of Tween-20. Seeds were then washed four times with sterilized distilled water and placed in Erlenmeyer flask (250 ml, Borosil), containing 50-60 ml half strength sterilized Murashige and Skoog medium (MS)⁶. Leaf, epicotyl and root explants were excised from 10-day-old seedlings, cut into 0.5-1 cm long pieces and surface sterilized with 0.1% HgCl_2 for 1-2 min followed by three washings with sterilized distilled water. The wet pieces were dried on sterilized blotting paper and inoculated on the callus induction media. Inoculations were done in laminar air flow chamber followed by incubation in an incubation room maintained at $25 \pm 1^\circ\text{C}$ and 16 hrs light and 8 hrs dark period having 700 lux light intensity. Five media, viz. MS, Linsmaier and Skoog (LS)⁷, Schenk and Hildebrandt (SH)⁸, Gamborg *et al* (B5)⁹ and White's (W)¹⁰, supplemented with 0.8% agar, 3% sucrose and 2,4-D, NAA and BAP 0.5 mg/l each were tested for callus induction. The pH of all media was adjusted to 5.8 before autoclaving at 121°C and 1.06 kg/cm^2 pressure for 20 min. To study the effect of different hormonal combinations on callus induction, 14 combinations of 2,4-D, NAA, IAA, BAP and Kin supplemented in MS medium were also tested. Periodic observations were made on callus initiation and morphology.

Shoot Regeneration

Five to six week-old calli were transferred to MS medium containing 8 combinations of NAA and BAP, 2% sucrose and 0.8% agar for differentiation and shoot regeneration. After 4-5 weeks of incubation, obtained shoot buds were subcultured on the same medium for multiple shoot development. Data on multiple shooting and number of shoots per shoot bud cultured were recorded.

Root Induction and Acclimatization of *in vitro* Derived Plantlets

Half strength MS medium supplemented with various combinations of growth hormones, viz. NAA, IBA and IAA, 2% sucrose and 0.7% agar was used to

initiate rooting in tissue culture derived shoots. *In vitro* grown shoots were separated from calli with the help of sterile blade, then placed vertically on the rooting medium and incubated in the growth chamber for root induction. Plantlets with well-developed root systems were removed from the medium. The roots were thoroughly washed with running tap water and plantlets were transferred to sterilized potting mixture of sand, soil and farm yard manure (1:1:1, w/w) in plastic pots and maintained for 10 days under highly humid conditions. Acclimatized plants were transferred to green house benches. Seeds were collected from these somaclones (R_0) upon maturity.

Evaluation of Pea Somaclones (R_1) for Disease Resistance

Somaclonal variation for disease resistance in pea was studied in R_1 generation against *A. pinodes* and *E. pisi* using detached leaf technique. For evaluation against *A. pinodes*, inoculum was prepared by harvesting pycnidia from freshly sporulating cultures in distilled sterilized water and spore concentration was adjusted to $3.0\text{-}5.0 \times 10^5$ spores/ml as measured by haemocytometer. Three leaves per plant were placed in Petri plates lined with moist blotting sheets and were drop-inoculated (10 μl) on the centre of leaves. Petri plates were incubated at $25 \pm 1^\circ\text{C}$ and data on disease development were recorded after 12 days of incubation. Reaction of somaclones to *A. pinodes* was categorized (on the basis of lesion size) as resistant (no infection), moderately susceptible (lesion ≤ 2 mm in diam) and susceptible (lesion ≥ 2 mm in diam).

Pure culture of *E. pisi* was established and maintained on Lincoln, a susceptible cultivar and 12-day-old culture was used for the evaluation of somaclones. Leaves from 30 to 40-days-old plants were detached and inoculated by dusting conidia on them using a camel hairbrush. Inoculated leaves were floated on sterilized tap water in 11 cm diam Petri dishes and incubated in a growth room maintained at $22 \pm 1^\circ\text{C}$, under 16/8 hrs light/dark period. After 12 days of incubation, the reaction type was scored as highly resistant, resistant, moderately resistant, susceptible and highly susceptible by using a 0-4 scale¹¹.

Enzymatic Assay

Isozyme analysis of resistant pea somaclones along with susceptible checks was carried out using peroxidase, esterase and phenylalanine ammonia lyase (PAL) enzyme systems. PAL was analysed through spectrophotometer whereas native-PAGE was performed for peroxidase and esterase isozymes. The

activity of PAL was determined following the method of Sadasivam and Manickam¹² with some modification. The enzyme was extracted by homogenizing 0.5 g of leaves in 1 ml of cold 25 mM borate buffer (pH 8.8) containing 5 mM 2-mercaptoethanol with pre-cooled pestle and mortar. The homogenate was centrifuged at 12000 g for 20 min at 4°C in a cooling centrifuge (Beckman, Avanti TM30, Rotor Type F 3602) and the supernatant served as enzyme source. PAL activity was assayed within 3 hrs of grinding the leaf tissues so as to avoid the loss in enzyme activity. Reaction mix was prepared using 300 µl aliquot of enzyme extract, 0.5 ml of borate buffer and 1.2 ml of water mixed in a test tube. The reaction was initiated with the addition of 1 ml of 0.1 M L-phenylalanine solution [165 mg L-phenylalanine (Biomark Laboratories, Pune) dissolved in 10 ml distilled water pH 8.7, adjusted with 0.1 N KOH]. Each treatment was repeated three times. The tubes were incubated at 32°C for 60 min in a water bath. The reaction was stopped by adding 0.5 ml of 1 M trichloroacetic acid. The contents in tubes were vortexed and absorbance was recorded at 290 nm in a VIS/UV spectrometer (Beckman, DU 640B). Cinnamic acid (Sisco Bioresearch Laboratories Pvt. Ltd., Mumbai) was used as a standard. The enzyme activity was expressed as µM cinnamic acid formed/min/mg protein.

For PAGE analysis proteins were extracted by grinding 500 mg leaf samples in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) with a precooled pestle and mortar. The homogenate was centrifuged at 12000 g for 15 min at 4°C in a cooling centrifuge. The extraction procedure was performed on ice and samples were also stored on ice or kept in fridge at 4°C. The protein content in the supernatant was determined by the method of Bradford¹³. The samples were prepared for native-PAGE in a sample buffer (0.062 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 10% sucrose and 0.002 % bromophenol blue) and concentration of protein in sample was adjusted to 0.5 µg/µl. An 80 µl of each sample containing 40 µg proteins was loaded into the wells. Electrophoresis was performed at 4°C at 150 V for 120 min in a vertical gel electrophoresis unit (Mini Model, Bangalore Genei Pvt. Ltd.) using 10% polyacrylamide resolving gel. Peroxidase isoforms were visualized by incubating the gels for 15 min in a solution containing benzidine (2.08 g), acetic acid (18 ml), 3% hydrogen peroxide (100 ml) and distilled water (80 ml) in dark. The reaction was stopped by placing the gel into a large volume of 7%

acetic acid for 10 min. For esterase analysis, gels were incubated for 1 hr in a solution containing disodium hydrogen phosphate (1.1 g), sodium dihydrogen orthophosphate (2.8 g), α-naphthyl acetate (0.03 g), fast blue RR salt (0.2 g) and water (200 ml) at 37°C in dark. The reaction was stopped by placing the gel into a large volume of methanol: water: acetic acid: ethyl alcohol (10: 10: 2: 1).

Statistical Analysis

The data obtained from PAL activity of both susceptible cultivars and resistant somaclones derived from them, callusing frequency in response to various hormonal combinations and callus differentiation were subjected to statistical analysis using CRD to study significance of difference in the treatments¹⁴.

Results and Discussion

Callus Induction

Five basal media, containing 2,4-D, NAA and BAP 0.5 mg/l each, were evaluated for callus induction from different explants of two pea cultivars, Lincoln and Palam Priya (Table 1). In case of Lincoln, 100, 93.3 and 80.0% callusing were observed on MS medium, respectively with leaf disc, epicotyl and root explants. Further, on B5 medium with leaf disc, epicotyl and root explants, the frequency of callus formation were 86.6, 93.3 and 73.3%. The good callusing in Lincoln was also observed on W medium with epicotyls (73.3) and leaf discs (66.6), LS medium with epicotyls (66.6) and SH medium with epicotyls (60.0%). Similarly, in case of Palam Priya, higher frequency of callus formation was observed on MS followed by B5 medium, except with root explant that showed lower response (53.3 and 46.6%) on both the media (Table 1). The good callusing in Palam Priya was also observed on W and LS media with epicotyl (66.6) and leaf disc (60.0%) explants, respectively. The overall callusing response in Palam Priya was, however, comparatively less than in case of Lincoln. The differential response of pea genotypes for callus induction has also been reported earlier^{15,16}. The callus induction frequency was found more in epicotyl and leaf as compared to root explants¹⁷. The calli produced from epicotyl tissues were compact, whereas those from leaf discs were friable¹⁸.

The effect of different hormonal supplements in MS medium was also studied for callus induction. Since MS medium was found the best of the five media tested for callusing, this was used as a basal me-

Table 1—Callus induction from different explants of pea on various media containing 2,4-D, NAA and BAP 0.5 mg/l each

Medium	Explant	Explant cultured (no.)	Lincoln		Palam Priya	
			No. of calli formed	Per cent calli	No. of calli formed	Per cent calli
SH	Leaf	15	3	20.0	7	46.6
	Epicotyl	15	9	60.0	6	40.0
	Root	15	6	40.0	4	26.6
LS	Leaf	15	8	53.3	9	60.0
	Epicotyl	15	10	66.6	8	53.3
	Root	15	7	46.6	3	20.0
MS	Leaf	15	15	100.0	12	80.0
	Epicotyl	15	14	93.3	13	86.6
	Root	15	12	80.0	8	53.3
B5	Leaf	15	13	86.6	11	73.3
	Epicotyl	15	14	93.3	12	80.0
	Root	15	11	73.3	7	46.6
W	Leaf	15	10	66.6	8	53.3
	Epicotyl	15	11	73.3	10	66.6
	Root	15	7	46.6	6	40.0

SH = Schenk & Hildebrandt⁸; LS = Linsmaier & Skoog⁷; MS = Murashige & Skoog⁶; B5 = Gamborg *et al*⁹; W = White¹⁰

dium for subsequent studies. In general, callus formation was more from leaf than epicotyl explants on the various hormonal combinations tested (Table 2). Hundred per cent callusing was achieved with leaf explants of Lincoln on MS medium supplemented with 2,4-D (5 mg/l), 2,4-D + NAA + Kin (1 mg/l each) and 2,4-D + NAA + BAP (0.5 mg/l each). These results confirm the previous finding, where 100% callusing in pea was also observed on MS medium containing 2,4-D, NAA and BAP (0.5 mg/l each)¹⁸. The callus formation was also high (90.0%) on medium supplemented with 2,4-D + NAA + IAA + BAP + Kin (0.5 mg/l each) for both leaf and epicotyl explants, and 2,4-D + NAA + BAP (0.5 mg/l each) for epicotyl explant of Lincoln. On the other hand, the highest callus induction in Palam Priya (90%) was observed on MS medium supplemented with 2,4-D + NAA + IAA + BAP + Kin (0.5 mg/l each), followed by 2,4-D + NAA + BAP (0.5 mg/l each) and NAA (1 mg) + Kin (0.2 mg/l) (86.6%) with leaf disc explants (Table 2). The successful callus induction in pea was earlier reported on MS medium containing 2,4-D (2-10 mg/l)¹⁹. However, the substitution of 2,4-D with IAA or NAA showed negative effect. Further, the calli formed on media containing higher amount of 2,4-D were compact, whereas those on NAA were friable.

Plant Regeneration

Of the seven combinations of NAA and BAP studied in MS medium for plant regeneration, only five gave morphogenic response. In both the cultivars, the calli originating from both leaf and epicotyl explants showed no callus differentiation on MS basal medium and also on the medium containing BAP 0.5 mg/l or NAA 0.5 mg/l + BAP 1.0 mg/l; however, medium containing rest of the combinations showed differentiation (Table 3). The frequency of callus differentiation increased with the increase in BAP concentration. The maximum calli differentiation was observed on medium containing BAP 6 mg/l for both the cultivars, and the minimum on BAP 2 and 4 mg/l for Lincoln and Palam Priya, respectively; the differences are statistically significant for Lincoln but not for Palam Priya.

Similarly, the highest number of shoots was obtained on MS medium containing BAP 6 mg/l for both the cultivars, and minimum on BAP 2 and 5 mg/l for Lincoln and Palam Priya, respectively, which are statistically different (Table 3). Though, maximum differentiation as well as maximum multiple shoots or shoot bud primordia was observed on MS medium supplemented with BAP 6 mg/l, the shoots formed withered within a few days and did not root either. On the other hand, the shoots formed on MS medium

Table 2—Effect of various hormones supplemented in MS medium on callus induction in pea*

2,4-D	Hormonal concentration (mg/l)			IAA	Explant	Callus formation (%)**	
	NAA	Kin	BAP			Lincoln	Palam Priya
0.2	2.5	1.5	-	-	Leaf	85.7 (69.78)	75.0 (60.00)
					Epicotyl	85.7(69.78)	66.6(54.70)
2.0	2.0	-	0.2	-	Leaf	60.0 (50.77)	66.6 (54.70)
					Epicotyl	40.0 (39.23)	33.3 (35.24)
1.0	1.0	-	-	-	Leaf	60.0 (50.77)	60.0 (50.77)
					Epicotyl	40.0 (39.23)	40.0 (39.23)
2.0	1.0	-	-	-	Leaf	40.0 (39.23)	60.0 (50.77)
					Epicotyl	40.0 (39.23)	55.5 (48.16)
-	2.0	-	0.2	-	Leaf	83.3 (65.88)	77.7 (61.82)
					Epicotyl	71.4 (57.67)	66.6 (54.70)
5.0	-	-	-	-	Leaf	100.0 (90.00)	76.9 (61.27)
					Epicotyl	57.1 (49.08)	60.0 (50.77)
2.0	2.0	-	0.5	-	Leaf	80.0 (63.43)	81.8 (64.75)
					Epicotyl	66.6 (54.70)	75.0 (60.00)
1.0	1.0	1.0	-	-	Leaf	100.0 (90.00)	73.3 (58.89)
					Epicotyl	71.4 (57.67)	66.6 (54.70)
-	1.0	2.0	-	-	Leaf	100.0 (90.0)	86.6 (68.53)
					Epicotyl	75.0 (60.0)	66.6 (54.70)
0.5	-	-	0.5	-	Leaf	81.8 (64.75)	72.7 (58.50)
					Epicotyl	71.4 (57.67)	75.0 (60.00)
-	2.0	-	1.0	-	Leaf	83.3 (65.88)	80.0 (63.43)
					Epicotyl	77.7 (61.82)	76.9 (61.27)
0.5	0.5	-	0.5	-	Leaf	100.0 (90.00)	86.6 (68.53)
					Epicotyl	90.0 (71.57)	80.0 (63.43)
0.2	0.2	0.2	-	-	Leaf	81.8 (64.75)	70.0 (56.79)
					Epicotyl	80.0 (63.43)	72.7 (58.50)
0.5	0.5	0.5	0.5	0.5	Leaf	90.0 (72.44)	90.0 (71.57)
					Epicotyl	90.0 (71.57)	80.0 (63.43)
CD (5%)						2.94	2.95

Values in parentheses are angular transformation; - = Not used; *Twenty-five explants were cultured in each treatment with three replications; **Average of three replications

containing BAP 2 to 5 mg/l were of good quality. These results are similar to the earlier study, where shoot formation was reported in calli cultured on MS medium supplemented with BAP 5 mg/l and NAA 0-4 mg/l²⁰. The differential behaviour of the genotypes for morphogenesis has also been reported in the study. Differentiation of calli and number of shoots obtained was more in Lincoln as compared to Palam Priya at various hormonal concentrations tested. Nauerby *et al*²¹ reported an increase in number of shoot buds formed with the increase in cytokinin concentration. Whereas, Jackson and Hobbs²² reported that cytokinin concentration above 5 mg/l could produce vitrified shoots, which were difficult to root. Similar results

are also obtained in the present investigation. *In vitro* flowering and seed set was also observed in the shoots, which remained on differentiation medium and were not transferred to fresh medium for rooting. Since aim of the study was to generate somaclones, seeds produced *in vitro* from differentiating calli were later used for raising R₁ generation. *In vitro* seed production can circumvent the problem of rooting in *in vitro* derived shoots and poor survival of somaclones in soil²³.

Root Induction and Transfer of *in vitro* Derived Plants to Pots

In general, rooting frequency was better on half strength MS medium containing NAA than IBA and

Table 3—Shoot regeneration in pea calli on MS medium supplemented with different concentrations of NAA and BAP*

Growth hormone (mg/l)		Lincoln**		Palam Priya**	
NAA	BAP	Callus differentiation (%)	Shoots/callus	Callus differentiation (%)	Shoots/callus
0.0	0.5	0.0 (0.33)	-	0.0 (0.33)	-
0.5	1.0	0.0 (0.33)	-	0.0 (0.33)	-
0.5	2.0	14.2 (22.14)	4.20	8.5 (16.95)	4.00
1.0	4.0	17.1 (24.43)	5.60	10.0 (18.43)	3.75
2.0	4.0	16.1 (23.66)	5.40	7.1 (15.45)	4.00
0.5	5.0	19.4 (26.13)	6.00	8.5 (16.95)	3.50
0.0	6.0	23.5 (29.0)	7.20	10.0 (18.43)	5.50
0.0	0.0	0.0 (0.33)	-	0.0 (0.33)	-
CD (5%)		5.08	0.93	4.30	0.84

Values in parentheses are angular transformation; - = Not recorded; *Thirty five calli were cultured in each treatment with two replications; **Average of two replications

Table 4—Root induction in *in vitro* derived shoots of pea on half strength MS medium supplemented with different hormones

Hormonal concentration (mg/l)			Lincoln		Palam Priya	
NAA	IBA	IAA	No. of shoots cultured	Per cent rooting	No. of shoots cultured	Per cent rooting
1.0	-	-	31	80.64	15	46.66
2.0	-	-	28	71.42	20	60.00
0.2	-	-	20	25.00	-	-
0.5	-	-	18	11.11	18	33.33
0.75	-	-	16	37.50	-	-
1.5	-	-	16	68.75	-	-
-	0.2	-	10	0.00	-	-
-	0.5	-	16	0.00	-	-
-	1.0	-	20	25.00	15	6.66
-	2.0	-	17	17.64	15	13.33
0.5	0.5	-	12	0.00	-	-
1.0	1.0	-	12	0.00	-	-
0.2	0.5	-	16	37.50	-	-
-	-	0.2	10	0.00	-	-
-	-	0.5	10	10.00	-	-
-	-	1.0	10	0.00	15	0.00
-	-	2.0	10	37.50	15	0.66

- = Not tested

IAA (Table 4). In Lincoln, the maximum rooting frequency was found in medium containing NAA 1mg/l (80.64), followed by NAA 2 mg/l (71.42) and 1.5 mg/l (68.75%). However, roots could not be induced on medium containing IBA 0.2 and 0.5 mg/l, IAA 0.2 and 1.0 mg/l, and NAA + IBA 0.5 and 1mg/l each. In Palam Priya, on the other hand, the maximum rooting (60%) was observed on half strength MS medium supplemented with NAA 2 mg/l, followed by NAA 1 mg/l (46.66%) and 0.5 mg/l (33.33%). However,

rooting frequency with IBA (6.66-13.33%) and IAA (0.66) was very low and roots could not be induced on IAA 1.0 mg/l. In a previous study, NAA 0.2 mg or IBA 0.4 mg/l have been reported to give good rooting results⁵. The high rooting frequency has also been reported on half strength B5 medium containing NAA 0.5 mg/l¹⁸. In the present study, the roots formed on medium containing NAA 2 mg/l were vigorous and survived better in soil than those obtained from NAA 1 mg/l. Shoots having node at cut end rooted better

and fast and in absence of node, callus formation took place and rooting was initiated from callus rather than from cut ends. In most of the cases single root initiated from the cut end followed by multiple roots. However, in certain cases on medium having NAA 2 mg/l, 2 or 3 roots were also initiated from cut end.

Of the 219 somaclones of Lincoln identified, only 43 survived in pots with a survival rate of 19.6%. The plants were kept under green house conditions till maturity and pod formation. The pod formation was observed in 31 of 43 plants. These fertile plants yielded only 1-3 pods/plant with 1-3 seeds/pod. In case of Palam Priya, 12 of 63 plants survived in pots with a survival rate of 19.0%. All the plants were fertile and yielded 1 to 2 pods/plant with 1-3 seeds/pod.

Reaction of Somaclones (R_1) to *A. pinodes* and *E. pisi*

Of the 71 R_1 plants derived from 31 R_0 plants of Lincoln, 5 somaclones (SL17, SL15, SL10, SL19-1 and SL25-1) were resistant, 16 moderately resistant and 50 susceptible to *A. pinodes* (Table 5). However,

none of the 71 plants tested showed any level of resistance to *E. pisi*. On the other hand, of the 21 somaclones of Palam Priya derived from 12 R_0 plants, only 1 (SP6-1) was found resistant, 6 moderately resistant and rest susceptible to *A. pinodes* (Table 6). The somaclone (SP6-1), resistant to *A. pinodes*, also showed moderate resistance to *E. pisi*; however, rest of the somaclones were either susceptible or highly susceptible to *E. pisi*. Besides, Sharma *et al*¹⁸ who have reported differences in spot size on 10 somaclones produced by *A. pinodes*, this is the first report on selection of resistant somaclone to *A. pinodes* and *E. pisi* through the use of somaclonal variations.

Isozyme Analysis

In the somaclones resistant to *A. pinodes*, the PAL activity (μ M of cinnamic acid formed/min/mg protein) was significantly higher than the susceptible checks. The highest PAL activity was recorded in SP6-1 (somaclone derived from Palam Priya) (Fig. 1). The pea plants were reported to show increased PAL

Table 5—Reaction of pea somaclones to *Ascochyta pinodes*

Reaction	Somaclones
Resistant	SL19-1, SL25-1, SL10, SL15, SL17, SP6-1
Moderately resistant	SL2-2, SL3-1, SL6-1, SL7-2, SL7-3, SL12-1, SL13-1, SL18-1, SL22-2, SL24-1, SL28-1, SL29-1, SL2, SL3, SL5, SL6, SP4-1, SP5-1, SP5-2, SP9-1, SP11-1, SP1
Susceptible	SL1-1, SL1-2, SL1-3, SL2-1, SL2-3, SL3-2, SL4-1, SL4-2, SL4-3, SL5-1, SL5-2, SL7-1, SL8-1, SL9-1, SL10-1, SL10-2, SL11-1, SL11-2, SL14-1, SL15-1, SL16-1, SL17-1, SL20-1, SL20-2, SL21-1, SL22-1, SL23-1, SL25-2, SL26-1, SL27-1, SL28-2, SL28-3, SL29-2, SL30-1, SL31-1, SL1, SL4, SL7, SL8, SL9, SL11, SL12, SL13, SL14, SL16, SL18, SL19, SL20, SL21, SL22, SP1-1, SP1-2, SP2-1, SP3-1, SP7-1, SP8-1, SP8-2, SP9-2, SP10-1, SP12-1, SP12-2, SP2, SP3, SP4, Lincoln, Palam Priya (checks)

SL= Somaclones of Lincoln; SP= Somaclones of Palam Priya

Table 6—Reaction of pea somaclones to *Erysiphe pisi*

Reaction	Somaclones
Highly resistant	-
Resistant	-
Moderately resistant	SL12-1, SL25-1, SP6-1
Susceptible	SL2-1, SL2-2, SL8-1, SL9-1, SL18-1, SL23-1, SL24-1, SL29-2, SL2, SL6, SL10, SL14, SL17, SL19, SL21, SL22, SP1-1, SP1-2, SP2-1, SP4-1, SP5-1, SP5-2, SP7-1, SP8-1, SP8-2, SP9-1, SP9-2, SP10-1, SP11-1, SP12-2, SP01, SP02, SP03, SP04, Palam Priya (check)
Highly susceptible	SL1-1, SL1-2, SL1-3, SL2-3, SL3-1, SL3-2, SL4-1, SL4-2, SL4-3, SL5-1, SL5-2, SL6-1, SL7-1, SL7-2, SL7-3, SL10-1, SL10-2, SL11-1, SL11-2, SL13-1, SL14-1, SL15-1, SL16-1, SL17-1, SL19-1, SL20-1, SL20-2, SL21-1, SL22-1, SL22-2, SL25-2, SL26-1, SL27-1, SL28-1, SL28-2, SL28-3, SL29-1, SL30-1, SL31-1, SL1, SL3, SL4, SL5, SL7, SL8, SL9, SL11, SL12, SL13, SL15, SL16, SL18, SL20, SP3-1, SP12-1, Lincoln (check)

SL= Somaclones of Lincoln; SP= Somaclones of Palam Priya

activity (50%) in response to pathogenic *Fusarium solani* f. sp. *pisi* compared to non pathogen *F. solani* f. sp. *phaseoli*²⁴. The varietal differences in pea have also been reported for the level of PAL²⁵. In the present investigation too, higher PAL activity was observed in Palam Priya (slow mildewing cultivar) than Lincoln. Increased PAL activity has also been reported in the rust and powdery mildew resistant lines than the susceptible Bonneville²⁶. These reports support the present findings.

The native-PAGE analysis for peroxidase activity in Lincoln and Palam Priya, and resistant somaclones derived from them is presented in Fig. 2. New isoform of peroxidase was observed in SP6-1 at Rf 0.26 (lane 7). The bands at Rf 0.20 and 0.31 were monomorphic but the band at Rf 0.31 was more intense in SP6-1 (Fig. 2a). In previous studies, two new iso-peroxidase bands have been observed in response to induced resistance in pea²⁷. Moreover, increased activity of anionic-peroxidase was reported in incompatible but not in compatible interactions²⁸. In the present study, variations in the esterase activity of Lincoln and Palam Priya, and their somaclone were also observed (Fig. 2b). The bands at Rf value 0.66 and 0.52 were monomorphic. However, a new isoform of esterase was observed at Rf 0.63 in SL25-1 (lane 20, a resistant somaclone of Lincoln). Three new isoforms were also seen in the somaclone of Palam Priya at Rf 0.33, 0.42 and 0.49 (lane 7), which were absent in Palam Priya (lane 6). New isoforms of esterase have also been detected in cotton in response to induced resistance against *Verticillium dahliae* by endophytic bacteria²⁹. The peroxidases and esterases, localized near the infected pea tissues, have been found to catalyze the formation of suberin³⁰. The suberization of cell walls around the infection site, presumably, blocks the progress of the pathogen and aids in the defence of the plant by forming a barrier.

In host defence mechanism, PAL is a key enzyme involved in the production of basic molecules used for biosynthesis of most of the phenolics, including phytoalexins and lignin³¹. PAL provides precursors for lignin and several phenylpropanoid derived secondary plant metabolites. The role of peroxidase appears in the final stages of lignin biosynthesis, which involves random dehydrogenative polymerization of precursors (cinnamyl alcohol) produced in phenyl propanoid pathway³². Hence, resistance observed in the somaclones could be because of the presence of any of these chemicals.

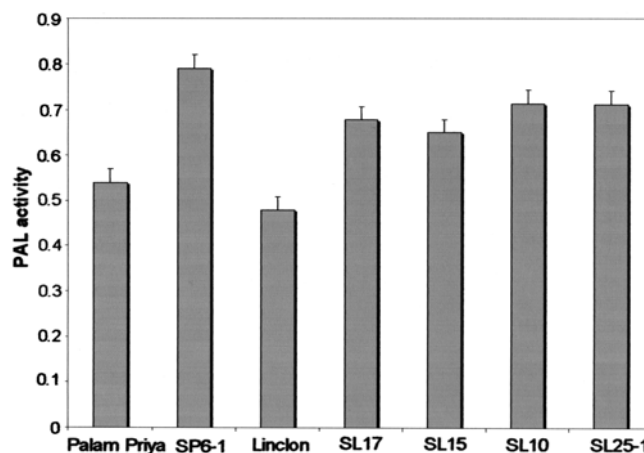


Fig. 1—Phenylalanine ammonia lyase (PAL) activity of pea somaclones resistant to *A. pinodes*. PAL activity expressed as μM of cinnamic acid formed per minute per milligram of protein. CD (5%) = 0.03

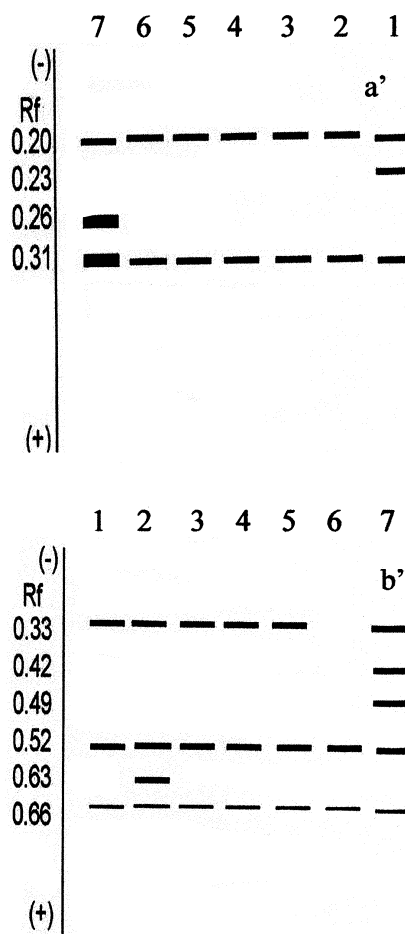


Fig. 2—Zymogram of pea somaclones resistant to *A. pinodes* for (a) peroxidase and (b) esterase isozymes. Lane 1-7: 1, Lincoln; 2, SL25-1; 3, SL10; 4, SL15; 5, SL17; 6, Palam Priya; and 7, SP6-1.

In conclusion, the protocol for the generation of pea somaclones has been standardized that can be evaluated for resistance against various biotic/abiotic stresses. The best medium for callus induction was MS supplemented with 2,4-D + NAA + BAP (0.5 mg/l each) using leaf explants as the calli obtained from leaf explants showed more morphogenic response when cultured on differentiation medium. MS medium containing NAA 0.5 mg + BAP 5 mg/l was good for regeneration. Vigorous rooting could be induced when *in vitro* derived shoots having node at cut end were cultured on half strength MS medium containing NAA 2 mg/l. One somaclone (SP6-1), resistant to *A. pinodes*, has been identified from Palam Priya, which also showed moderate resistance to *E. pisi*. Resistance to *A. pinodes* was also observed in five somaclones of Lincoln. Increased PAL and peroxidase activity was found in SP6-1, which further affirms the role of these enzymes in disease resistance.

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