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SHORT COMMUNICATION

STUDIES ON *IN VITRO* REGENERATION AND DIRECT ORGANOGENESIS IN PEA (*PISUM SATIVUM* L.)

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A regeneration protocol has been optimized in Pea (*Pisum sativum* L.) genotypes HUDP-15 and IPF-99-25 with explants viz. embryonic axes as such, and also as sliced and decapitated and cotyledonary node. Direct shoot regeneration was achieved on medium containing MS salts + B_5 vitamins and various concentrations of BAP, NAA and IBA. The best direct regeneration efficiency (10.37±3.18 shoots/ explant) and frequency (84.66±0.84%) was achieved from embryonic axes slices on MS salts + B_5 vitamins + 3.0 mg/l BAP + 2.0 mg/l IBA + 40 gm/l sucrose. The regenerated shoots were excised and transferred to rooting medium, containing NAA and IAA at various concentrations. The highest rooting was observed on the medium supplemented with ½ MS salts + B_5 vitamins + 2.0 mg/l IAA + 30 gm/l sucrose, rooted plants were acclimatized and transferred to field with good survival rate.

Key words: **IBA, *in vitro*, pea, regeneration**

Pea (*Pisum sativum* L.) is an important crop in the Indian sub-continent. It is grown as a source of protein for human diet. Pea, like most other grain legume species is recalcitrant to genetic modification, although protocols of regeneration and transformation of pea have been described (Bean *et al*. 1997). However, these systems have drawbacks such as low efficiency as well as high frequency of escapes and the occurrence of chimeric genetically modified plants. Polowick *et al*. (2000) reported the regeneration of pea from different parts of the plant, either by organogenesis (Gamborg *et al.* 1974) or by somatic embryogenesis (Lehminger–Mertens and Jacobsen 1989). These regeneration systems are indirect because they include an intermediary callus phase which delays organogenesis and yielding plants with somaclonal variation. Somatic embryogenesis from immature embryos have been reported (Flandre *et al.* 1989) but long period of time which elapses before the shoots or embryo appears (4-5 weeks) to make these systems less suitable for transformation.

The introduction of foreign genes into pea plants have been delayed by the lack of suitable regeneration protocol. It has been previously shown that pea is susceptible to infection by several strains of the *Agrobacterium tumefaciens* (Hobbs *et al*. 1989). The main objective of this study was to standardize *in vitro* regeneration system and to work out relative effect of plant growth regulators, medium, genotypes and explants. The present communication reports an efficient *in vitro* regeneration protocol suitable for genetic transformation.

Pea genotypes viz. HUDP-15 and IPF-99-25 obtained from Co-ordinating unit MULLaRP, Indian Institute of Pulses Research (IIPR), Kanpur were used

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as a source of plant material for regeneration. Different explants viz. embryonic axes, embryonic axes slices, cotyledonary nodes and decapitated embryonic axes were used for standardization of regeneration protocol. The seeds were soaked overnight in flask containing 25 ml of sterile distilled water. Soaked seeds were sterilized in 70% (v/v) ethanol (1 min) followed by 10% sodium hypochlorite (15 min) and three washes with sterilized water.

Explants for regeneration were cut from the embryonic axes of these seeds. Seed cultures were initiated by aseptically transferring 25 explants in each petri plate (20 ml medium). The basal culture medium contained MS macro and micronutrients (Murashige and Skoog 1962), B_5 vitamins (Gamborg *et al*. 1968), and 40 g sucrose. Agar was added as a solidifying agent and media were poured with growth regulators, auxins (NAA, IAA) and cytokinins (BAP) in different combinations. All media were adjusted to pH 5.6 prior to sterilization by autoclaving (120^{0}C, 15 psi). IAA and NAA were filter sterilized and added to the autoclaved medium just prior to pouring into petri plates. The cultures were kept in culture room at temperature of 24±1^{0}C and relative humidity (RH) of 65%±2ºC. The photoperiod light/dark cycle were maintained as 16/8 hours respectively. The light intensities (through white fluorescent tubes) given to cultures were 3500 lux.

Swelling in explants started at 8 d and became larger within 12 d (Fig. a, b). After 12 d, these swollen explants started showing bud formation (Fig. b). The explants needed subculturing after every 10 days which increased the shoot number (Fig. c, d). The multiple shoots were separated and transferred to medium containing high concentration of cytokinins (BAP) which promoted shoot elongation within 15 d (Fig. d, e). The elongated shoots (Fig. e, f) were transferred to a rooting medium and after 20 to 22 days, rooting was observed in the shoots. The well rooted shoots (Fig. g, h) were taken out, washed thoroughly and transplanted into plastic pots containing mixture of sand, soil and vermiculite (Fig. i). The combination of MS salts and B_5 vitamins in the medium was proved to be the best medium (Frequency, 96.00±1.57 and efficiency, 10.37±3.18) under present investigation (Table 1). Pniewski *et al.* (2003) also found

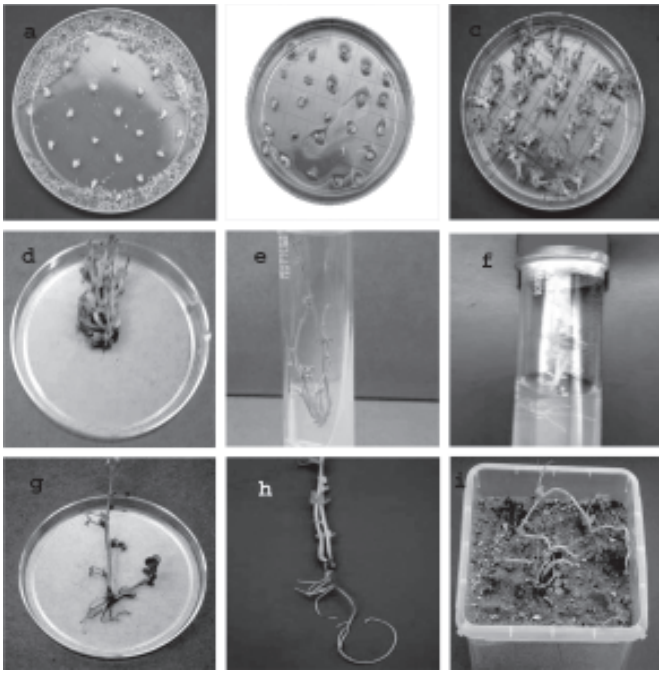

Fig. 1. *In vitro* regeneration of Pea (*Pisum sativum* L.), a. Embryonic axes slice explant b. Multiple shoot induction c. Proliferation of shoots d. Elongated shoots e. *In vitro* flowering of elongated shoots f. Rooting of elongated shoots g. Shoots with profuse rooting h. Elongated shoots with rooting i. Plants transferred to plastic pot.

combination of MS salts and B_5 vitamins as the best medium for regeneration of pea. However, Lim and Song (1987), Kysely *et al.* (1987) and Tetu *et al*. (1990) reported MS as better medium for regeneration of Pea. Among various combinations of auxins and cytokinins used (Table 1), maximum efficiency (10.37±3.18 shoots/ explant) was obtained on medium containing 3.0 mg/l BAP and 2.0 mg/l IBA. However, the other treatments generally showed little or poor response to growth regulators. Maximum frequency (96.00±1.57) was obtained on medium containing 2.0 mg/l BAP + 2.0 mg/ l IBA followed by MS salts + B_5 Vitamins + 2.0 mg/l BAP + 1.75 mg/l NAA (94.00±2.14%) and MS salts + B_5 Vitamins + 3.0 mg/l BAP + 2.0 mg/l IBA (90.22±0.42%). However, other treatments showed moderate response to different growth regulators (Table 1). Effect of genotypes was observed for both frequency as well as efficiency of shoot induction among the genotypes. HUDP-15 genotype showed better response (90.22±0.42) to regeneration followed by IPF-99-25

Table 1. Effect of medium and PGR on *in vitro* regeneration in pea genotypes.

Hormones (mg/l)			HUDP-15		IPF-99-25	
BAP	**NAA**	**IBA**	**Frequency ± SE**	**Efficiency ± SE**	**Frequency ± SE**	**Efficiency ± SE**
1.00	-	-	77.33±0.64	1.06±0.79	-	-
2.00	-	-	82.00±7.71	4.57±3.30	-	-
3.00	-	-	82.00±1.59	2.18±1.98	-	-
4.00	-	-	71.00±0.6	1.50±1.20	-	-
2.00	1.75	-	94.00±2.14	3.36±2.08	94.00±0.80	4.46±1.03
2.00	2.00	-	79.33±1.38	4.09±3.07	78.66±0.98	4.94±2.18
2.50	0.50	-	81.75±0.52	6.39±1.34	79.60±1.30	5.05±3.20
2.00	-	2.00	**96.00±1.57**	2.42±1.5	72.00±0.73	3.87±1.68
3.00	-	2.00	90.22±0.42	8.66±3.04	84.66±0.84	**10.37±3.18**
3.00	-	2.50	83.50±0.64	5.91±3.97	88.00±1.27	3.63±2.61

**Above data were recorded every 10 days and every treatment repeated 8-10 times*

(84.66±0.84). IPF-99-25 was found to be more efficient (10.37 + 3.18 shoots/explant) than HUDP-15 (8.66±3.04) (Table 2). Further, widest range of shoots/explant was found with genotype IPF-99-25 (12-15) followed by HUDP-15 (10-12).

The nature of explants plays an important role in regeneration response both in term of frequency as well as efficiency. Embryonic axes exhibited best shoot regeneration frequency (90.33±0.8%) while embryonic axes slices exhibited best shoot regeneration efficiency (6.87±1.91 shoots/explant) (Table 3). Elongated and well developed shoots were excised from the shoot clumps and transferred to ½ MS medium augmented with various concentrations of NAA, IAA for root initiation (Table 4). Rooting occurred in 20 days. The frequency of rooting varied with different auxin concentrations used (Table 4). The medium containing 2.0 mg/l IAA produced best root induction (frequency, 89.2±1.07% and efficiency, 3.68 ± 2.13 roots/shoot) followed by medium containing 1.0 mg/l NAA (frequency, 76.05±1.56% and

Table 2. Differential effect of genotype on *in vitro* regeneration in pea (*Pisum sativum* L.).

Genotype	Frequency ± SE	Efficiency ± SE
HUDP-15	90.22±0.42	8.66±3.04
IPF-99-25	84.66±0.84	10.37±3.18

Table 3. Effect of explants and genotypes on *in vitro* regeneration in pea (*Pisum sativum* L.).

Explant	Genotype			
	HUDP-15		IPF-99-25	
	Frequency ± SE	**Efficiency ± SE**	**Frequency ± SE**	**Efficiency ± SE**
Embryonic axes	**90.33±0.8**	5.45±4.6	78.78±2.1	4.67±0.7
Embryonic axes slice	82.92±1.5	3.70±0.91	86.25±1.2	**6.87±1.91**
Decapitated embryonic axes	82.00±1.98	6.82±1.74	88.00±0.9	4.28±1.80
Cotyledonary node	56.00±1.41	2.19±0.70	74.00±1.85	3.1±2.5

Table 4. Effect of auxins on rooting of *in vitro* regenerated shoots of pea (*Pisum sativum* L.)*.

Auxins (mg/l)	Frequency ± SE	Efficiency ± SE
NAA		
0.5	73.10±1.36	1.57±1.49
1.00	76.05±1.56	1.89±2.05
IAA		
0.5	81.48±1.11	0.86±1.07
1.00	38.88±1.21	1.51±2.01
2.00	**89.2±1.07**	**3.68±2.13**

efficiency, 1.89±2.05 roots/shoot). About 70-75% of well rooted plantlets exhibited establishment in the green house within 20 days after transplanting. The pea regeneration protocol described here would be of immense use for genetic transformation studies.

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