Plant Cell Reports (1987) 6:305–308

Plant Cell Reports

Plant regeneration via somatic embryogenesis in pea (*Pisum sativum* L.)*

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Received March 5, 1987 / Revised version received May 22, 1987 – Communicated by O. L. Gamborg

ABSTRACT

Whole plant regeneration via somatic embryogenesis was obtained in pea (Pisum sativum L.) using explants from immature embryos or shoot apex segments. The induction of somatic embryos required picloram or 2,4-D. Germination of fully-developed embryos was accomplished by subculture on medium with only cytokinin and then on medium supplemented with cytokinins in combination with a reduced auxin concentration. Plantlets obtained from both zygotic embryos and shoot apices were transferred to soil and were grown to maturity. Nine plants were examined cytologically, revealing three tetraploids (2n=4x=28) and six diploids (2n=2x=14).

ABBREVIATIONS

Picloram, 4-amino-3,5,6-trichloropicolinic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthaleneacetic acid; BA, 6-benzylaminopurine; IBA, indole-3-butyric acid

INTRODUCTION

The pea (Pisum sativum L.) has been regenerated from complex explants via in vitro organogenesis (Mroginski and Kartha, 1984, for a review; Devreux, 1970; Gamborg et al., 1974; Atanassov and Mehandjiev, 1979; Malmberg, 1979; Mroginski and Kartha, 1981; Rubluo et al., 1984; Hussey and Gunn, 1984; Kunakh et al., 1984; and Ezhova et al., 1985), but plant regeneration via in vitro embryogenesis has not been previously accomplished. Embryogenic suspension cultures were established from leaf- and stem-derived callus in a liquid medium containing picloram (Jacobsen and Kysely, 1984). Attempts to induce shoot morphogenesis from these embryos have been unsuccessful (Jacobsen and Kysely, 1984; Kysely, 1985). Interrupted development of embryo-like structures has been reported in suspension cultures of other grain legumes such as Phaseolus (Martins and Sondahl, 1984; Allavena and Rossetti, 1983), Vigna (Bhargava and Chandra, 1983) and Glycine (Beversdorf and Bingham, 1977; Phillips and Collins, 1981; Gamborg et al., 1983).

Regeneration of Glycine max from immature cotyledons via somatic embryogenesis was first reported by Lippmann and Lippmann (1984). Routine regeneration systems are now established for this species using immature cotyledons with or without the embryonic axis as an explant source, (Lazzeri et al., 1986; Ranch et. al., 1985; Barwale et al., 1986). In pea, the induction of embryo-like structures from mature cotyledons was previously observed (E. Cunningham, Dept. of Agronomy, Univ. of Kentucky, personal communication). These structures, however, did not develop into plantlets.

We report the first whole plant regeneration via somatic embryogenesis in pea from immature zygotic embryos and from the youngest node of shoot apex segments.

MATERIALS AND METHODS

Three pea genotypes were grown in a growth chamber under a 16/8 hour light/dark and a 24°C/15°C day/night temperature regime, (white fluorescent lamps, 78-110 $\mu Em^{-2}s^{-1}$). The genotypes were MN 494-A11 (provided by E.T. Gritton, University of Wisconsin, Madison, WI, USA); R4111b, a recombinant derived from a cross between the fasciated mutant 489C and 'Dippes Gelbe Viktoria' (Lönnig, 1982); and a line of Pisum sativum var. arvense (provided by Dr. S. Blixt, Weibullsholm Plant Breeding Experiment Station, Landskrona, SWEDEN).

Immature pods were surface-sterilized by immersion for 30 sec in 70% 2-propanol alcohol followed by a 12 min immersion in 25% commercial sodium hypochlorite solution (Chlorox) with a few drops of detergent (Liquinox) and subsequently, two rinses in sterile deionized water. Embryos ranging in size from 0.5 to 9.0 mm were excised from their seed coats and placed on media containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 3% sucrose, 0.7% Phytagar (Gibco) and supplemented with phytohormones as listed in Table 1. The medium was adjusted to pH 5.8 before autoclaving. A total of 403 embryos with seven per petri dish (100x20 mm, Falcon) were cultured. Dishes were incubated at 25±3°C under diffuse cool white fluorescent or Grolux (Sylvania) light (520 $\mu Em^{-2}s^{-1}$) with a 16 hour photoperiod.

To obtain shoot apices, seed of P. sativum var. arvense was surface-sterilized as described above except that treatment times of 1 min in 70% 2-propanol alcohol and 15 min in 25% Chlorox were used. Seed was germinated on agar-solidified SGL medium (Phillips and Collins, 1985). Shoot apices 5mm long and including the youngest node from five-day-old etiolated pea seedlings were placed on modified MS medium (as above), supplemented with 0.5, 1.0 or 5.0 mgl^{-1} picloram and cultured under the conditions specified above. Thirteen, 20, and

* KAES Journal Article No. 87-3-4
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18 apices were cultured on 0.5, 1.0, and 5.0 mgl^{-1} picloram, respectively.
Somatic embryos induced on either immature embryo or shoot apex cultures, were transferred to MS medium (as above) containing 1.0 mgl^{-1} BA, and subsequently to MS medium, but containing 0.05 mgl^{-1} NAA and 0.017 mgl^{-1} each of BA, kinetin and zeatin (Lazzeri et al., 1987). "Germinated" somatic embryos were rooted on either 1/2 B5 salts (Gamborg et al., 1974) with 2% sucrose and 0.2 mgl^{-1} NAA or HPN medium (salts as Eskew et al., 1983; with 0.25 mgl^{-1} $NiSO_4 \cdot 6H_2O$, B5 vitamins, 2% sucrose, 0.65% phytagar at pH 5.9 before autoclaving; Lazzeri et al., 1987) with 0.25 mgl^{-1} IBA. Both media were contained in GA7 vessels (Magenta Corp., Chicago), and cultures were placed under 30 $\mu Em^{-2}s^{-1}$ cool white fluorescent light with a 16 hour photoperiod and day/night temperatures of 20/15°C. Rooted plantlets were transferred to a 3:2, peat:sand mixture in pots in a greenhouse, where they were grown to maturity. Chromosome counts were made on root tips of nine regenerated plants.

Root tips collected for chromosome counts were pretreated in 4 mM 8-hydroxyquinoline for 5-6 hr at 4°C. Enzymatic digestion of root tips was carried out in a solution of 0.5% each cellulase RS (Onozuka) and pectolyase Y-23 (Sheishin). They were then fixed for 15 hr in 3:1 (95% ethanol:glacial acetic acid) at room temperature. Root tips were rinsed with water, then stained with feulgen and squashed on a glass slide in acetocarmine.

RESULTS AND DISCUSSION

Immature Embryo Cultures

Shoots and roots of immature embryos elongated when cultured on media with 0.001 mgl^{-1} picloram or 5.0 mgl^{-1} NAA, while embryos callused when cultured on media with 1.0 and 0.05 mgl^{-1} picloram or 1.0 and 0.5 mgl^{-1} 2,4-D. Cotyledons of embryos <3 mm senesced after 3 to 6 d of culture, and callus was derived only from their embryonic axes. After 25 to 35 d of culture, somatic embryos of different sizes could be observed on explants from R4111b and P. sativum var. arvense (Figure 1) whereas cultures of MN 494-A11 produced only a few globular structures on medium with the highest picloram concentration. Explants from R4111b produced somatic embryos on all media except 0.001 mgl^{-1} picloram and 5.0 mgl^{-1} NAA. Immature zygotic embryos of P. sativum var. arvense were plated only on 0.05 mgl^{-1} picloram medium. Somatic embryos derived from R4111b and P. sativum var. arvense on picloram containing media developed trumpet-shaped or normal cotyledons, in comparison with embryos formed on 2,4-D media which often remained in the globular stage. Somatic embryos were obtained from zygotic embryos which were 2.0 to 9.0 mm in length.

Genotypic effects for growth in vitro and regeneration ability have been reported in pea (Malmberg, 1979; Jacobsen et al., 1980; Rubluo et al., 1984; Hussey and Gunn, 1984; Ezhova et al., 1985; Kysely, 1985). Our results suggest that there may also be differences in frequency of somatic embryogenesis from immature embryos among the three genotypes used in this study.

Among hormone treatments eliciting response, 5 to 15% of embryos plated produced somatic embryos with an overall success of 12% (Table 1). Among auxin treatments, picloram at 1.0 mgl^{-1} and 2,4-D at 0.5 mgl^{-1} was most efficient for production of somatic embryos, but conversion of embryos into plants was less efficient for 2,4-D than picloram. Approximately two somatic embryos for every responding zygotic embryo were obtained from all treatments (Table 1). Regeneration was obtained from primary cultures only. It may be possible through subculture of morphogenetically competent tissues, to produce continuously regenerating cultures.

Shoot Apex Cultures

Shoot apices bleached, enlarged and formed slow growing callus when cultured on media with 1.0 or 0.5 mgl^{-1} picloram. Those explants on 5.0 mgl^{-1} picloram exhibited only cell enlargement and browning of the tissue. Embryo-like structures emerged from the axillary-bud meristem region after 20 to 35 d of culture. These embryo-like structures had very similar morphology to somatic embryos derived from immature embryos, and were distinct from leaf structures produced via organogenesis. When detached from the parental tissue and subcultured, the radical often elongated. No embryo-like structures were produced by shoot apices cultured on 5.0 mgl^{-1} picloram, however, 7 of thirteen explants on 0.5 mgl^{-1} and 4 of twenty on 1.0 mgl^{-1} picloram showed a positive response. Picloram has also been found to be highly effective in the induction of somatic embryo-like structures in suspension culture of pea (Jacobsen and Kysely, 1984) and soybean (Gamborg et al., 1983).

Maturation and Rooting

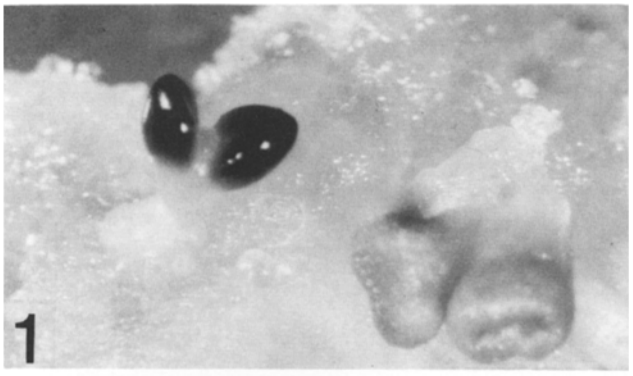
All somatic embryos failed to undergo further development when subcultured on induction medium. Transfer of embryogenic cultures to MS medium with 1.0 mgl^{-1} BA stimulated development of young embryo stages but generally repressed shoot outgrowth (Figure 2). After one month, somatic embryos were then transferred to MS medium with 0.05 mgl^{-1} NAA and 0.017 mgl^{-1} each of BA, kinetin and zeatin for further development. Similar requirements have been observed for maturation of soybean somatic embryos (Lazzeri et al., 1985; Barwale et al., 1986). Generally, several shoots emerged from the shoot apex region of the embryos but some germinated in exactly the same manner as zygotic embryos (Figure 3). Germinated somatic embryos or shoots ≥2 cm in length were transferred for rooting to either 1/2 B5 medium with 0.2 mgl^{-1} NAA or HPN medium with 0.25 mgl^{-1} IBA. Rooted shoots from both immature embryos and shoot apices were transferred to soil (Figure 4) and were grown to maturity. Approximately 15 plants are currently in soil or have produced seed. Five of these plants were from shoot apex cultures while the remainder arose from immature embryos. Chromosome counts on root tips of nine plants showed six to be diploid (2n=2x=14) and three to be tetraploid (2n=4x=28) (Figure 5). All tetraploids originated from apex cultures and in one case, both tetraploid and diploid shoots arose from the same explant.

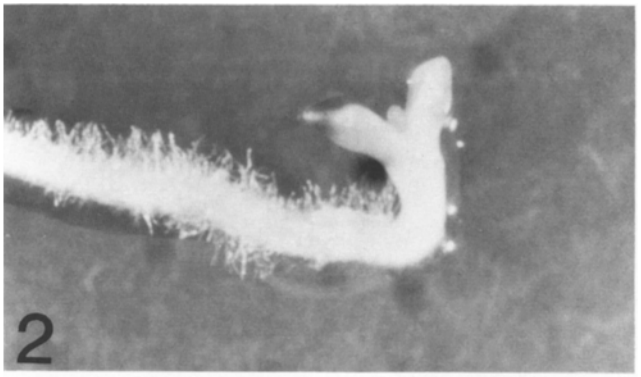
Further investigations with immature embryo cultures will determine whether regeneration involves a truly dedifferentiated phase or occurs from meristematic tissues of the embryonic axis. Our observations to date, however, indicate that somatic embryos can arise from cotyledon tissue quite removed from the embryonic axis.

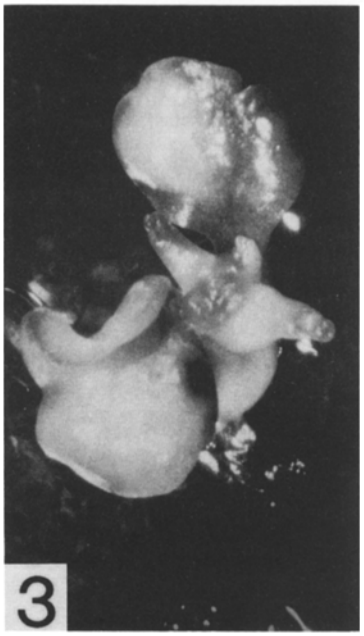
The present results suggest that picloram can shift axillary-bud meristems from organogenesis to somatic embryo-like development. The developmental process by which this takes place deserves investigation.

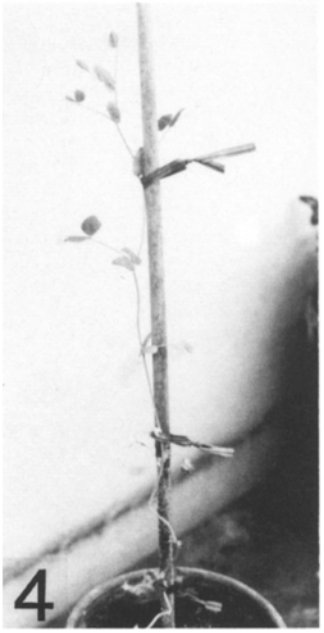
Table 1: Effect of auxin on somatic embryogenesis from immature zygotic embryos of three genotypes of pea.

Auxin (mgl^{-1})		Zygotic Embryos Plated	Responding		Somatic Embryos Total	Per zygotic embryo
		---------- no. ----------		%	---------- no. ----------	
Picloram	1.0	91	14	15	41	2.9
Picloram	0.05	55	3	5	5	1.7
Picloram	0.001	69	0	0	0	0.0
2,4-D	1.0	57	6	11	13	2.2
2,4-D	0.5	54	8	15	12	1.5
NAA	5.0	77	0	0	0	0.0
Total (Responding)		257	31	12	71	2.1









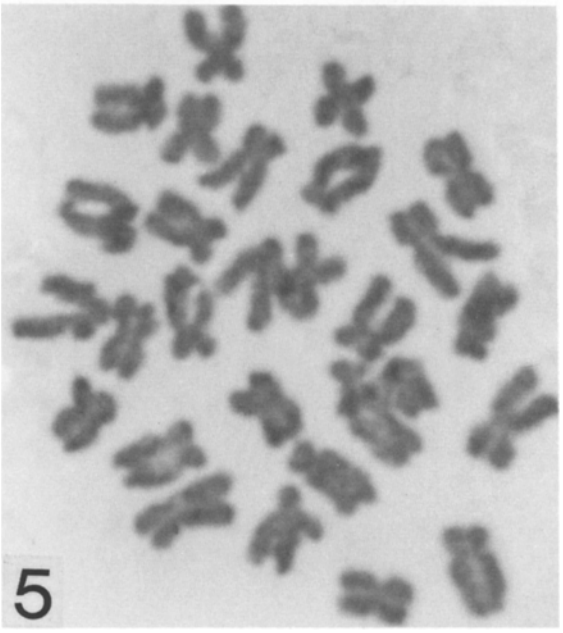


Figure 1: Somatic embryos from immature zygotic embryo of P. sativum var. arvense, induced on medium with 0.05 mgl^{-1} Picloram. Two have trumpet-shaped cotyledons while a third has normal cotyledons.

Figure 2: Root outgrowth from an isolated somatic embryo (see Figure 1) on medium with 1.0 mgl^{-1} BA.

Figure 3: "Germination" of an isolated somatic embryo from a zygotic embryo (genotype R4111b) cultured on medium with 1.0 mgl^{-1} BA.

Figure 4: Potted plant of P. sativum var. arvense from an embryo-like structure from an axillary-bud meristem.

Figure 5: Root tip squash from P. sativum var. arvense shoot apex-derived plant showing tetraploid (2n=4x=28) chromosome number.

ACKNOWLEDGEMENTS

This work was made possible by a fellowship from the DAAD to W. Kysely and a grant of the Bundesministerium für Forschung und Technologie, FRG, to H.-J. Jacobsen. The authors gratefully acknowledge the technical assistance of J. Sunega, chromosome preparations by S. Berger and manuscript preparation by I. Deaton and A. Fahrenwald.

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