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NATURE AND MODE OF REGENERATION IN COTYLEDONARY NODAL EXPLANTS OF PIGEONPEA

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SUMMARY

***In vitro* shoot regeneration and histological studies were conducted to understand the nature and mode of regeneration in cotyledonary nodal explants of pigeonpea (*Cajanus cajan* (L.) *Millsp.)* cv. Pusa 855 under the influence of BAP. It was observed that presence of pre-existing axillary bud was absolutely necessary for the multiple shoot bud induction and regeneration involved direct organogenesis without any intermediary callus phase. Dedifferentiation occurred from the sub-epidermal cells of the basal parts of pre-existing axillary bud.**

***Key words* : Cotyledonary node, pigeonpea, regeneration.**

INTRODUCTION

Pigeonpea (*Cajanus cajan* (L.) *Millsp.)* is one of the major grain legume crops of the semi-arid tropics and subtropics. Pigeonpea seeds are a rich source of protein (24%) and form an important protein supplement to Indian vegetarian diet. Productivity of pigeonpea remained stagnant for many years, being limited by biotic stresses. Conventional breeding methods were largely less productive because of various linked undesirable characters like small, hard and bitter seeds (Reed and Lateef 1990). Consequently, in order to broaden the existing gene pool it becomes essential to exploit biotechnological tools and techniques to introduce foreign genes from inaccessible sources into the genetic background of cultivated species. An efficient *in vitro* regeneration system is, however, necessary for application of biotechnological methods. Cotyledonary nodal culture appears to be a very successful method in the *in vitro* shoot regeneration protocols used for legumes (Somers *et al.* 2003) including pigeonpea. Successful genetic transformation requires an indepth understanding of which cells undergoing regeneration in which explant tissue and at what stage of development of the explant under consideration (van Wordragen and Dons 1992). It is in this context, studies were conducted on regenerating cotyledonary nodes of pigeonpea to ascertain the nature and mode of regeneration.

MATERIALS AND METHODS

Pigeonpea cv. Pusa 855 seeds obtained from Division of Genetics, Indian Agricultural Research Institute, N. Delhi, were used in the present study.

In vitro regeneration: The protocol developed by Rao (1997) was used in the present study with minor modifications. Healthy seeds were washed with diluted laboratory detergent for 2-3 minutes and rinsed with tap water to remove all traces of the detergent. Seeds were then sterilized with 0.1% mercuric chloride for 5 minutes and later rinsed with autoclaved distilled water 4-5 times in the laminar air flow chamber. Sterilized and mercuric

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chloride free seeds were then inoculated on autoclaved liquid ¼ B5 media (Gamborg *et al.* 1968) supplemented with 10 µM BAP, with absorbent cotton as the supporting material for the seeds. Cotyledonary nodes with intact seed from 4-day-old seedlings were excised aseptically and used as explants. Explants were inoculated on to solidified B5 medium supplemented with 10 µM BAP (shoot bud induction medium), putting the epicotyl end down towards the medium.

To study the nature of regeneration, the pre-existing axillary bud of cotyledonary node was either left undisturbed or carefully removed/nipped (to remove apical meristem only) before inoculating on the shoot bud induction medium and regeneration response was observed. The histology of the regenerating explants was also studied using microtomy.

Microtomy: Longitudinal sections of regenerating explants of cotyledonary nodes (8 µM thick) were prepared with rotary microtome having steel blades, using the paraffin embedding procedure as described by Johansen (1940). Sections were stained with 0.5% aqueous hematoxylin and micro-photographed at 100x magnification.

RESULTS

Presence of pre-existing axillary bud is necessary for regeneration from the cotyledonary nodal explants of pigeonpea, as there was no multiple shoot bud induction from the explants with pre-existing axillary buds completely removed, though there was callus production (Fig. 1 A). It was also found that removal of only apical meristem of pre-existing axillary bud did not affect the multiple shoot bud regeneration to any great extent from pigeonpea cotyledonary nodal explants (Fig. 1 B).

Fig. 1. Effect of axillary bud on regeneration response of cotyledonary nodal explants of pigeonpea (Photographs taken at 15 DAI) A. Axillary bud completely removed, B. Axillary bud apical meristem removed, C. Axillary bud undisturbed

Histological studies of the regeneration events revealed that at two days after inoculation (DAI) there were no dedifferentiating cells in explants (Fig. 2 A, B). Initiation of dedifferentiation was observed in 4 DAI explants, precisely at and around the basal part of the pre-existing axillary buds, in the sub-epidermal regions (Fig. 2C, D) as evident from the densely stained, isodiametric cells, denoting actively forming meristematic regions. By the 7th DAI, meristematic region expanded and initials of multiple shoots can be observed in the histological sections (Fig. 2 F). The duplicate samples presented in Fig. 2 show that there were differences in regeneration response of different explants-differences in axillary bud size at 2 DAI (Fig. 2 A, B) and at 4 DAI (Fig. 2 C, D) and difference in meristem enlargement at 7

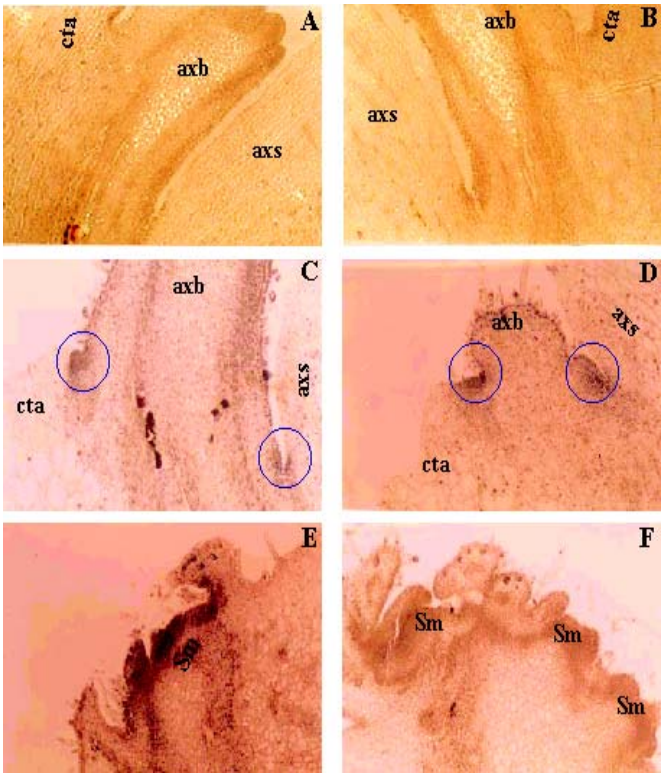

Fig. 2. Longitudinal sections of cotyledonary nodal explants of pigeonpea showing nature and mode of regeneration. A&B: 2 DAI, C&D: 4 DAI, E&F: 7 DAI, axb: Preexisting axillary bud, cta: Cotyledonary attachment tissue, axs: axis of seedling, sm: shoot meristem, Encircled portions in C and D show the dedifferentiating cells at the base of preexisting axillary buds

DAI (Fig. 2 E, F). In Fig. 2 E, it was clearly observed that under culture, pre-existing axillary bud shortened with broadened apical meristem, which would give multiple shoot initials on further culture. It was also clear from these histological studies, that there was no intervening callus phase in multiple shoot bud production in cotyledonary nodal explants of pigeonpea, since there was no unorganized tissue (callus) development in regenerating explants. Evidently multiple shoot regeneration from BAP cultured cotyledonary nodal explants of pigeonpea was through direct organogenesis from *de novo* dedifferentiating cells of basal parts of pre-existing axillary buds.

DISCUSSION

Complete removal of pre-existing axillary bud totally eliminated the regeneration response and that the removal of only apical meristem of pre-existing axillary bud gave regeneration comparable to that of normal cotyledonary nodal explants (Fig. 1). Further it became evident that the presence of axillary buds was required, with only basal portions of the same being essential for multiple shoot bud induction from cotyledonary nodal explants of pigeonpea. The results also revealed that only callus development occurred when axillary buds were completely removed from the cotyledonary nodes (Fig. 1). In essence it is the combination of presence of BAP and pre-existing axillary bud, rather the basal portion of bud, that are required for multiple shoot bud induction from the cotyledonary nodes of pigeonpea. The present findings are contradictory to the findings as reported by Prakash *et al.* (1994), where it has been shown that complete removal of pre-existing axillary buds from cotyledonary nodal explants of pigeonpea doesn't affect the multiple shoot bud regeneration. It is quite possible that while removing the axillary buds all the dedifferentiating cells of basal portions of axillary buds might not have been removed as the explants were prepared from 12 days old seedlings which were already showing a mass of shoot initials at cotyledonary nodes. Maintenance of morphological integrity of cotyledonary nodal explants in legumes to obtain multiple shoot production as emphasized by Malik and Saxena (1992a, b, c) was in line with the present findings, but applicable only up to the basal portions of pre-existing axillary buds rather than the whole. Formation of meristematic tissue at the base of pre-existing axillary bud that in turn gave rise to multiple buds under the influence of cytokinins was observed in *Phaseolus* (Franklin *et al.* 1991, Malik and Saxena 1992 a, b).

Histological studies showed that multiple shoot bud induction from cotyledonary nodal explants involved direct organogenesis from pre-existing axillary buds and initiation of dedifferentiation of cells was observed at 4 DAI in sub-epidermal regions of basal parts of pre-existing axillary buds (Fig. 2). Though the time and location of initiation of dedifferentiation of tissue was not noted, direct organogenesis from cotyledonary nodes was reported through histological studies in chickpea (Malik and Saxena 1992c, Murthy *et al.* 1996), *Phaseolus* (Malik and Saxena 1992a, b), and lentil (Malik and Saxena 1992c) and through direct observations in pea (Griga *et al.* 1986), *Phaseolus* (McClean and Grafton 1989, Franklin *et al.* 1991), and in pigeonpea (Franklin *et al.* 1998), which are in accordance with the present findings. However, oranogenesis through callus from cotyledonary nodes as reported by Sarangi and Gleba (1991) in pigeonpea embryonic axis explants obtained from germinating seeds and from split cotyledonary nodes obtained from mature seeds as observed by Kumar *et al.* (1983, 1984) are against the present findings. In these studies the callus development was probably due to the injuries caused during explants preparation, and multiple shoot buds developed from the pre-existing axillary bud, rather from the callus itself.

In conclusion, the present study indicates that regeneration from cotyledonary nodal explants in pigeonpea involves direct organogenesis from the sub epidermal regions of basal parts of pre-existing axillary bud.

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