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Histological Study of Callus Formation and Root Regeneration from Mung Bean (*Vigna radiata* W.)

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We have established a reproducible culture system for callus formation and root development from juvenile stem segments of mung bean (*Vigna radiata*). In particular, we have studied the influence of plant growth regulators. Induction of calli from young stem explants was very effective on MS inorganic salts supplemented with 0.5 mg/L 2,4-D and 1.0 mg/L kinetin. In regenerating adventitious roots from callus tissues, we found that a combination of 0.75 mg/L NAA, 1.5 mg/L kinetin, and MS salts resulted in 20% efficiency. Histological examination showed that callus tissues originated from out-growths of the cambium rings through de-novo meristematic activity. Those rings were localized outside the vascular cambium. Adventitious roots that developed from root primordia originated from the center of the Callus masses. These primordia produced tracheid-like cells, which then became meristemoid cells for the cambium. Newly formed adventitious roots had the typical tetrarche actinostele type.

Keywords: meristemoid, organogenesis, rooting, *Vigna radiata*

Extensive research has been conducted on organogenesis in plant tissue culture ever since adventitious root and shoot inductions were first reported from the calli of carrot (Nobecourt, 1939) and tobacco (White, 1939). Anatomical and histological studies of organ differentiation have been reported for many species, using different explant types. During organogenesis, callus-derived adventitious organs originate from three regions of the cambium: external phloem, internal phloem, and callus tissue (Sterling, 1951).

Adventitious roots develop from inside the callus because of the influence of shoots preformed within the callus cells (Paterson and Rost, 1981; Gladfelter and Phillips, 1987; Wagley et al., 1987). However, it has also been reported that independent of those effects from the shoots, the meristematic nodules are induced from serial divisions of certain cells within the callus under special culture conditions, and differ from the adventitious roots (Steward et al., 1958). Soh et al. (1980) have shown that root primordia from the adventitious roots of *Phaseolus vulgaris* develope from divisions within the vascular cambial cells and phloem parenchyma. The derivatives of those primordia then penetrate the bundle sheath and cortex, finally elongating toward the epidermis or cork cells. However in peanuts, the adventitious roots arise from the epidermal cells rather than, the vascular cambium (Atreya et al., 1984).

As evident from these conflicting reports, the origins of adventitious shoots and roots, as well as the developmental process for these two organs during organogenesis, have not yet been established. Different explant types and culture conditions can result in various pathways. Although many papers have been published on cytodifferentiation and root development (Haissig, 1988; McCown, 1988), the results have been confusing and sometimes contradictory because nearly every study has dealt with different species at several developmental stages, all tested under widely varying experimental conditions (Haissig, 1986; Gonzalez et al., 1991).

The objective in this study was to determine, the optimum conditions for callus induction and adventitious root formation from young stem explants of mung bean (*Vigna radiata* W.). Moreover, we used cytohistological observations to evaluate the callus origin, the initial cells of the adventitious roots, and the process for their formation.

MATERIALS AND METHODS

Plant Material

Seeds of mung bean (*Vigna radiata* W.) were planted in flats containing commercial potting-mix soil, and germinated in a growth chamber.

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Callus Induction and Organ Development

Seven-day-old stems were surface-sterilized with a 2% sodium hypochlorite solution for 10 min. After rinsing three to four times with sterile distilled water, 2-mm-long segments were placed on Murashige and Skoog (1962; MS) inorganic salts. Calli were induced in the dark at 25°C, then subcultured to fresh MS inorganic salts every four to five weeks. The MS medium was supplemented with 30 g/L sucrose, 100 mg/L myo-inositol, 10 g/L agar, and various concentrations and combinations of 2,4-D, IAA, and NAA (auxins) as well as kinetin and, BAP (cytokinins). Adventitious roots were induced later from calli that were maintained in a root-inducing medium.

Cytohistological Observations

Young stem explants and calli grown for 12, 24, 36, 48, 60 or 72 h, or for 5 or 7 d following subculture, were histologically examined. The adventitious roots that differentiated from the callus tissue at two, three, and four weeks after culture were fixed in a formalin-acetic acid-alcohol (FAA) solution at room temperature for 12 h. After dehydration in an alcohol series, they were transferred to xylene and embedded in pure paraffin. The paraffin blocks were sectioned using a rotary microtome set at 10 μm thick. After being double-stained with hemalum and safranin, the sections were observed and photographed using light microscopy.

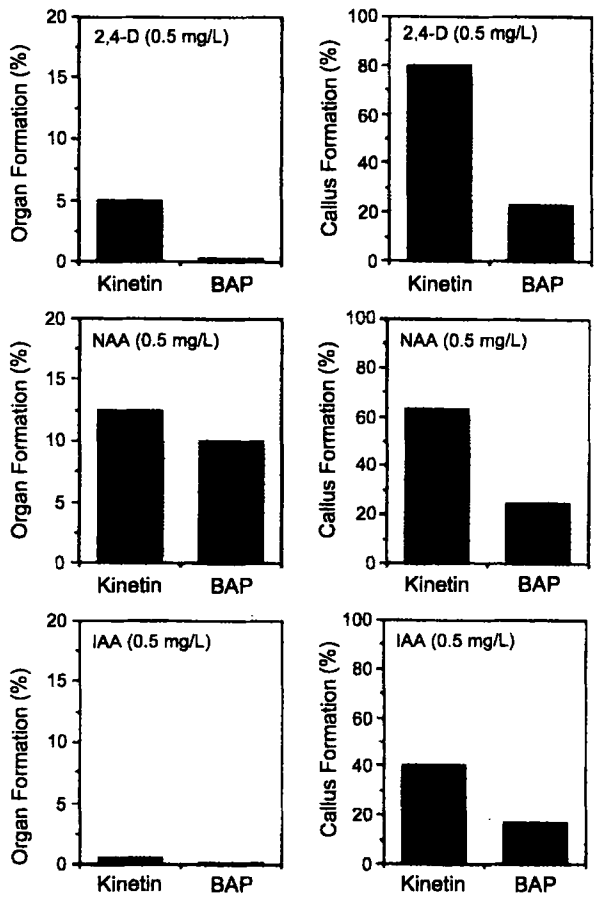

Figure 1. Effects of various auxin and cytokinin combinations on callus and root formation from the young stem of *V. radiata* after four weeks of culture.

RESULTS

Optimized Media for Callus Induction and Organ Development

The most effective combination of growth regulator concentrations for callus induction was 0.5 mg/L 2,4-D and 1.0 mg/L kinetin in an MS medium (Fig. 1). However, media supplemented with 0.75 mg/L NAA and 1.50 mg/L kinetin produced better results for root regeneration from callus (Fig. 2).

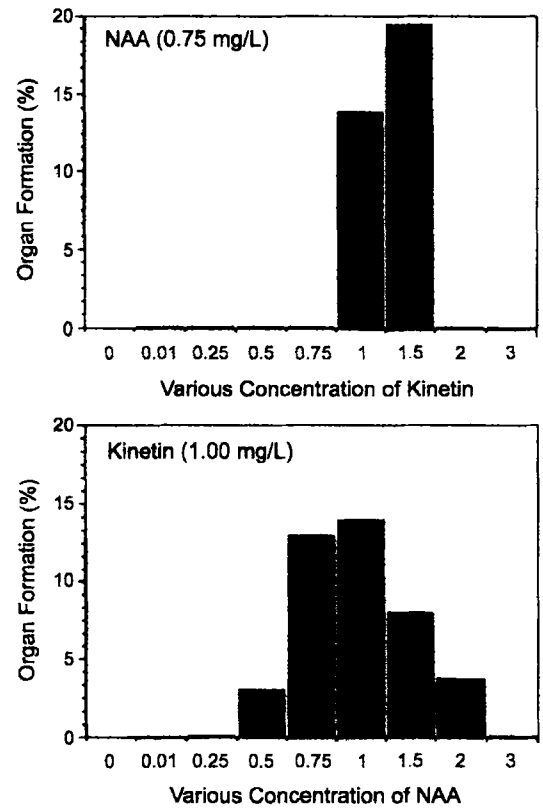

Figure 2. Effects of various concentrations of NAA and kinetin on root formation from the young stem of *V. radiata* after four weeks of culture.

Origin of Callus Formation

Calli were induced from young stems 4 to 5 d after culturing in an MS medium containing 0.75 mg/L NAA and 1.50 mg/L kinetin (Fig. 3A). The explants were fixed and sectioned every 12 h after culture. By that time, the parenchyma cells from the cortical tissue located in the outer phloem were enlarged and elongated more than those in control stems (Fig. 3, B and C). After 24 h of culture, a few cells along these elongated cortical parenchyma and epidermal cells had initiated periclinal and anticlinal divisions, thereby forming a

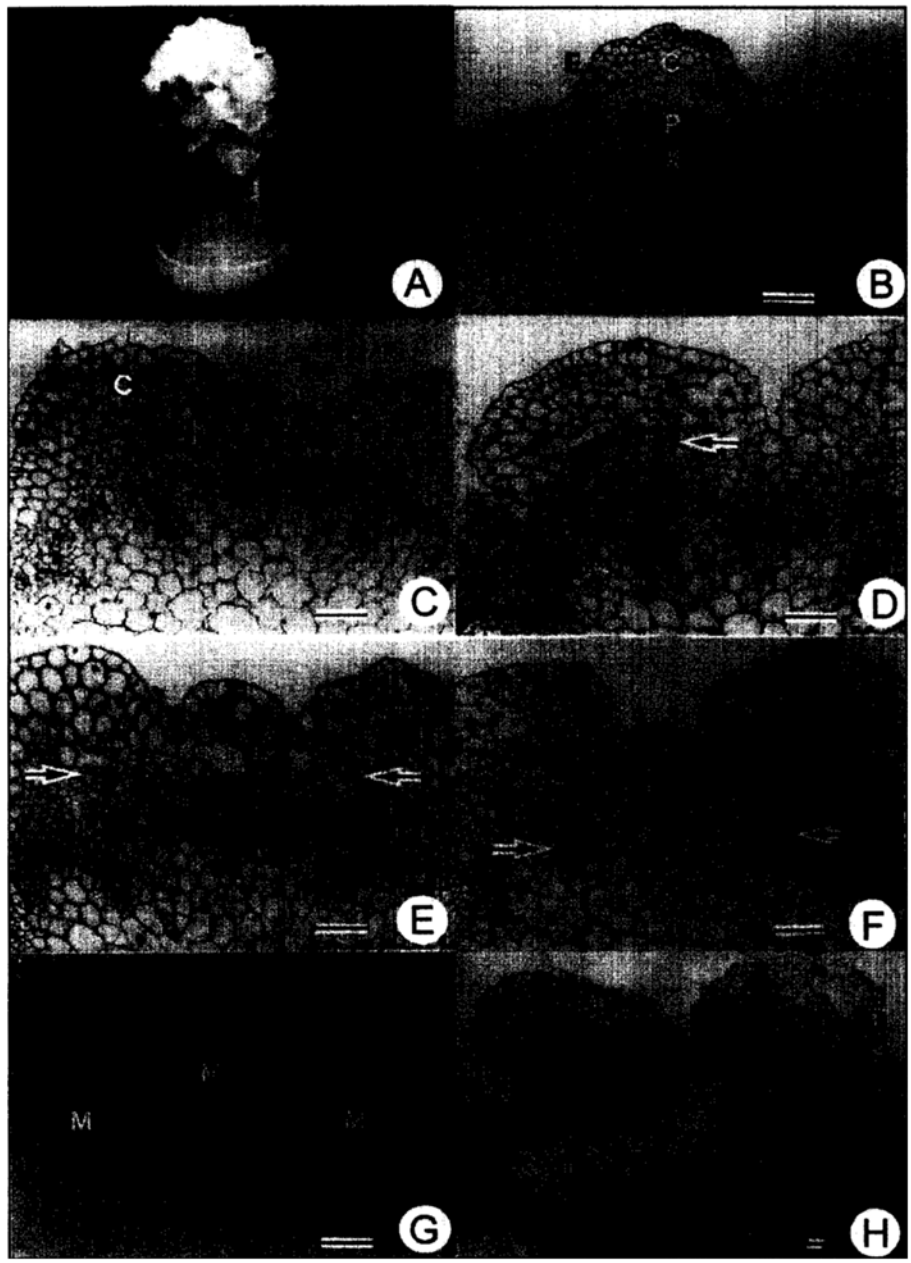

Figure 3. Transverse sections of cultured stem and calli in various developmental stages. **A.** Callus produced from young stem of *V. radiata* after four weeks of culture. **B.** Internal structure of control stem. **C.** Enlarged cortex parenchyma after 12 h of culture. **D.** Cambium-like structure (arrow) formed after 24 h of culture. **E.** Callus cambium ring (arrow) formed after 36 h of culture. **F.** Meristematic nodules (arrow) initiated from vascular parenchyma cells. **G.** Meristematic nodule (M) formed after 48 h of culture. **H.** Callus (Ca) tissue formed after 72 h of culture. C, cortex; E, epidermis; P, phloem; Pi, pith; X, xylem. Scale bar = 100 μm.

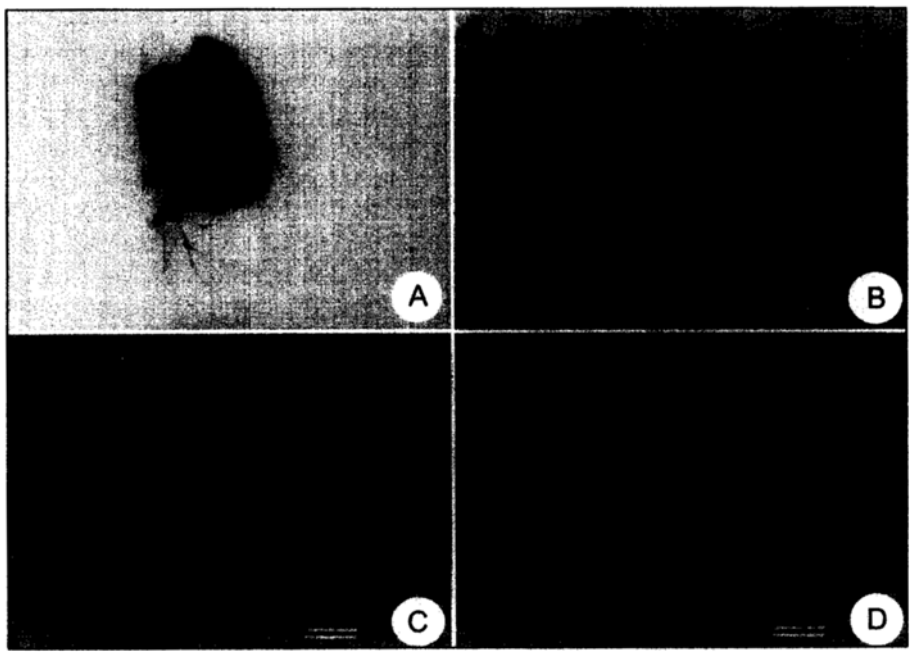

Figure 4. Transverse and longitudinal sections of adventitious roots from *V. radiata* in various developmental stages. **A.** Adventitious roots produced from callus after six weeks of culture. **B.** Adventitious root primordia initiated from meristematic nodule. **C.** Root primordium formed after two weeks of culture. **D.** Apical meristem of adventitious root. Scale bar = 100 μm.

cambium-like structure (Fig. 3D). After 36 h, phloem parenchyma cells of the vascular bundle, vascular bundle cells, and pith cells were elongated and starting to divide. By then, most of the stem-tissue cells had converted to cambial cells. The pre-existing vascular bundles disappeared at this stage, and meristematic cells formed on the outside of the vascular bundle. These bundles were connected to each other by the formation of a cambium-like zone (e.g., interfascicular or cork cambiums), which developed during secondary growth (Fig. 3E). After 48 h, a newly-formed cambium-like zone from the perivascular tissue had divided anticlinally and periclinally, so that calli originated from these derivatives of the cambial cells. In addition, meristematic nodules initiated from the vascular parenchyma cells (Fig. 3F). These nodules were observed at various places in the newly formed callus, and de-novo tracheal elements were found at their centers (Fig. 3G). After 72 h, vigorous outgrowths were observed from the callus mass (Fig. 3H).

Origin of Adventitious Roots

White cells protruded from the surface of the callus tissue two weeks after culture, when the young stems were placed on MS media supplemented with 0.75 mg/L NAA and 1.50 mg/L kinetin. After six weeks of culture, these protuberances developed into adventitious roots (Fig. 4A). We used light microscopy to investigate this process of development. In the calli that were cultured for two weeks, the initial cells of the adventitious-root primordia arose from a meristematic nodule (Fig. 4B). Roots then developed from those primordia that had differentiated from the nodules (Fig. 4C). Origins of the root primordia were scattered in the callus cells. The internal structures of the adventitious roots were actinostele (very similar to a normal root stele), and were also clearly visible in the apical meristems of the root tips (Fig. 4D).

DISCUSSION

The efficiency of adventitious-shoot or root development from callus varies according to the combination of auxin and cytokinin applied to the media (Flick et al., 1983). Although cytokinins stimulate cell division, if the optimal ratio of auxin to cytokinin is supplied, this combination enhances not only division but also cell differentiation (Boulter and Crocomo, 1979). In general, organogenesis in higher plants is controlled by manipulating that balance of growth hormones (Skoog

and Miller, 1957). However, in some leguminous crops, such as cowpea, shoot morphogenesis is not often so easily regulated (Gill et al., 1987).

For our mung bean, the MS medium supplemented with 0.75 mg/L NAA and 1.50 mg/L kinetin was the best for promoting organ differentiation from the induced calli. Crocomo et al. (1976a) studied a kidney bean tissue-culture system, and found that, although 2,4-D was very effective for inducing callus growth, it was not beneficial in adventitious-root regeneration. However, further experimentation showed that if, instead of using 2,4-D, the culture medium was supplemented with 10 mg/L NAA and 10 mg/L kinetin, adventitious root regeneration was much more successful (Crocomo et al., 1976b). Evans at al. (1976) also reported that an MS medium supplemented with 10.75 M NAA and 2.33 M kinetin was very effective in promoting root formation from the calli of *Glycine max*.

Adventitious roots did not develop from calli placed in media without sugar and auxin. The efficiency of root formation also is significantly decreased when the cultures are grown in the light (Liau and Boll, 1970; Olieman-van der Merr et al., 1971). Peters et al. (1976) have reported that regeneration efficiencies are very low in a kinetin-free medium, and that root formation is significantly enhanced in media supplemented with IAA, kinetin, and 50 mg/mL nicotine as alkaloids.

Figure 5 provides a summary diagram for the various stages observed during the regeneration process. In our study, calli developed from the excised stem fragments, originating from the cambium cells that were formed from the connections of actively dividing

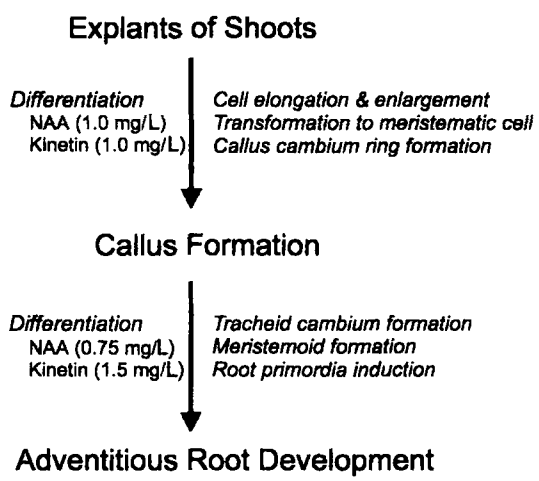

Figure 5. Simplified scheme illustrating the essential features of serial differentiation during callus induction and root development from shoot explants of *V. radiata*.

meristematic cells. These meristematic cells were converted from elongated and enlarged cortical parenchyma and epidermal cells of the excised explants. Concurrently, most of the explant cells, including the vascular bundle and pith, became meristematic and started to divide. Adventitious roots that formed from the callus developed from root primordia that were derived from cambium-tracheid cells. The latter region had arisen from the meristemoids, including the tracheids and scattered cambium, on the inside of the callus. We also noted that the root primordia developed from the tracheid-cambium formed on the inside of the callus tissue, and that the adventitious roots originated from the peripheral regions of the tracheid elements. These developmental steps have also been reported by Halperin (1966), Nuti Ronchi (1981), Davies et al. (1982), Howarth et al. (1983), and Anzidei et al. (1996).

The stages of adventitious root development from the excised young stem fragments of mung bean can be subdivided into two steps: 1) dedifferentiation (explants to callus), and 2) redifferentiation (callus to root primordia via callus cambium-ring formation) (see Esau, 1977). Our schematic is very similar to that described for the cotyledon fragments of *Corylus avellana*. There, Gonzalez et al. (1991) found that some of the parenchyma cells divided actively, producing meristematic centers that later developed into root primordia and adventitious roots. Likewise, Gautheret (1969) showed that the excised rhizome fragments of *Helianthus tuberosus* produced roots in vitro through a sequence of morphogenetic phenomena: 1) proliferation and differentiation of phloem and tracheids, 2) organization of the cambium, and 3) formation of root primordia from the cambium. This process is similar to that for shoot development from calli.

Regeneration of adventitious shoots was promoted by differentiation of the meristemoids that had originated on the surface of the calli. In contrast, primordial root formation is induced by differentiation from the meristemoid formed on the inside of the callus (Villalobos et al., 1985; Gladfelter and Phillips, 1987; Wagley et al., 1987). The pattern of meristemoid formation from callus varies case by case in shoots and roots (Torrey, 1966), and little is known about the processes of physiological and biochemical differentiation. Moreover, investigations are necessary to determine which meristemoids differentiate into shoots or into roots, and under what specific culture conditions.

Because knowledge is limited about the organogenesis process, researchers are challenged in trying to identify the few cells that are involved in callus organ-

ogenesis, as well as separating them from cells that are non-embryogenic or non-meristemoid cells. Another problem is that calli may lose their potential for organogenesis because the processes of adventitious shoot and root development are not induced simultaneously. Finally, one must test which factors are affected by the differentiation of the meristemoid for shoot or root regeneration.

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