PLANTLET FORMATION IN BLACK AND WHITE SPRUCE. III. HISTOLOGICAL ANALYSIS OF IN VITRO ROOT FORMATION AND THE ROOT-SHOOT UNION

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(Received for publication 12 December 1985; revision 12 August 1986)

ABSTRACT

The present study describes the histology of adventitious root formation on the **in vitro**-formed shoots of **Picea mariana** (Mill.) B.S.P. and **P. glauca** (Moench) Voss (black and white spruce). Some swelling occurred at the base of the shoot, and some callus was formed below the base under the influence of indole-3-butyric acid (IBA), an active ingredient of the rooting powder used. Some of the cells within the base of the shoot and in the vicinity of the vascular system differentiated into cambium-like cells which later produced tracheid nests and resin canals. The tracheid nests were composed of irregularly arranged tracheids of various sizes surrounded by the cells of the cambium. Some of the derivatives of the cambial cells located at the periphery of the tracheid nests differentiated into the root meristemoids. The cells of these meristemoids were small and contained densely staining cytoplasm and large nuclei. Later in culture, these cells differentiated into the root primordia which then assumed the normal configuration of a root. These roots were connected with the vascular tissue of the shoots through the tracheid nests. The continuity of the vascular system of the tissue culture-derived plantlets of black and white spruce was confirmed by clearing them with sodium hydroxide and then staining.

Keywords: tracheid nests; root meristemoids; adventitious roots; **Picea mariana**; **Picea glauca**.

INTRODUCTION

In vitro micropropagation of forest trees is emerging as an alternative to asexual propagation through rooted cuttings. *In vitro* plantlet formation has been reported in several important forest tree species in the last decade (David 1982; Mason & Mayne 1984; Dunstan & Thorpe 1986). Three distinct phases of plantlet formation have been recognised: (1) initiation of shoot buds on the explant; (2) development and elongation of shoots; and (3) rooting of shoots. Histological and histochemical changes leading to initiation and development of adventitious shoots in various conifer explants have been the subjects of several of our studies (e.g., Patel & Berlyn 1983; Patel & Thorpe

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1984; Villalobos *et al.* 1985; Rumary *et al.* 1986; Thorpe & Patel 1986). However, the understanding of root initiation leading to *in vitro* plantlet formation is superficial and based mainly on observations of seedling cuttings (e.g., Smith & Thorpe 1975; Montain *et al.* 1983a, b). Several nutritional, hormonal, and physical factors influence the rooting process in forest trees (David 1982). For example, in *Pinus pinaster* Sol., naphthalene acetic acid concentrations influenced the quality of roots formed, and at 10^{-6} M induced not only many roots, but also callus at the base of the shoots which often prevented the establishment of a vascular connection between the roots and shoots (Rancillac 1979).

This study describes the histological changes leading to adventitious root formation on the tissue culture-derived shoots, and the anatomy of the root-shoot union in the *in vitro*-regenerated plantlets of black and white spruce.

MATERIAL AND METHODS

The epicotyl explants of black and white spruce were prepared and cultured in Schenk & Hildebrandt's (SH) medium (1972) as reported earlier (Rumary & Thorpe 1984). After about 4 months of culture the shoots elongated enough (at least 1 cm) to be used for rooting. The bases of the shoots were dipped in autoclaved "Stim-root No. 1" rooting powder (active ingredient 0.1% IBA) and placed into sterile vermiculite soaked in half-strength SH mineral salts containing 0.2% charcoal. The medium lacked organic supplements except 2% sucrose. The trays with shoots were then incubated in a growth chamber at 20°C day/18°C night temperature under 12-h photoperiod (at a photon fluence rate of c. 50 μmol/m^2/s PAR). Maximum root formation was achieved after about 37 days of the treatment; but root initiation was asynchronous and some shoots rooted in 8 days. Hence, several shoots were selected for histological analysis after 8 and 37 days of the rooting treatment. The shoots were fixed in FPA (formalin, propionic acid, 50% ethanol in 5 : 5 : 90 proportion) for up to 8 h, dehydrated in a TBA series, embedded in Paraplast, and sectioned at 10 μm. The sections were stained with safranin-fast green (Jensen 1962) and azure B (Flax & Himes 1952). The root-shoot union was observed after clearing the plant material in sodium hydroxide and staining with safranin-basic fuchsin-crystal violet (Gardner 1975).

RESULTS AND DISCUSSION

The anatomy of the basal region of a tissue culture-derived shoot of white spruce is shown in Fig. 1. The central core of pith was surrounded by the vascular tissue and the cortex was composed mainly of parenchyma. The basal regions of the needles can be seen as domes at the periphery of the cortex. Sections of black spruce shoots had a similar appearance.

Distinct morphological and histological changes were visible 8 days after the rooting treatment in both spruces. The base of the shoot became swollen and a small amount of callus was formed at the base. In conifer cuttings, this basal callus has been shown to originate from various tissues of the central cylinder and the cortex (Satoo 1955; Reines & McAlpine 1959; Heaman & Owens 1972). In a transverse section, the shoot base showed a variety of cells and tissues (Fig. 2). The central xylem cylinder increased considerably in size, presumably owing to the increased activity of the cambium. The

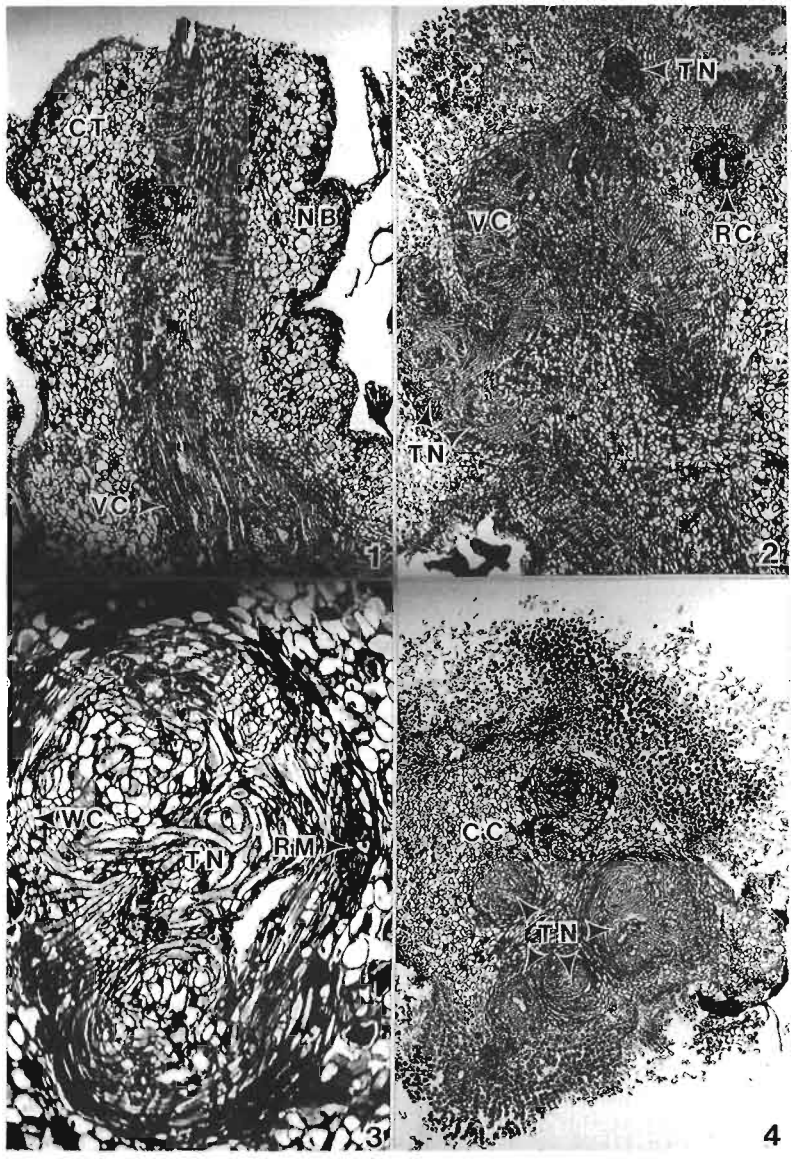

FIG. 1–4 — Anatomy of root initiation in adventitious shoots of white spruce. Fig. 1: Longitudinal section of the base of the shoot, showing the vascular cylinder (VC), cortex (CT), and the needle bases (NB) (×115). Fig. 2: Transverse section of the shoot base cultured for 8 days, showing formation of tracheid nests (TN) and resin canals (RC) among the basal cells near the vascular cylinder (VC) (×115). Fig. 3: Magnified view of a tracheid nest (TN) showing irregularly arranged tracheids surrounded by wound cambium (WC). Note the formation of a root meristemoid (RM) from the cambial cells located at the periphery of the tracheid nest (×415). Fig. 4: A transverse section at the junction of the shoot base and the basal callus showing several tracheid nests (TN) among the unorganised cell mass (CC) (×65).

vascular cylinder was surrounded by loosely arranged parenchyma cells in the cortical region. Many of the cells in the swollen basal region in the spruces accumulated large amounts of tannin and other ergastic substances. Some of these cells were devoid of such substances and differentiated into cambium-like cells, which later produced tracheid nests, in addition to well organised resin canals (Fig. 2).

In *Pinus banksiana* Lamb. seedling cuttings, several kinds of tissues were found to differentiate within the base and callus, viz vascular cambium, secondary xylem, secondary phloem, periderm, and resin canals (Montain *et al.* 1983b). In the present study, in addition to these tissues, the development of tracheid "nests" was also observed. Most of these nests developed very close to the vascular tissue of the shoot (Fig. 2). This supports our assumption that it was cells near the original vascular cambium of the shoot which differentiated into the wound cambium-like cells. The tracheid nests were composed of irregularly arranged tracheids of various sizes surrounded by cells of this wound cambium (Fig. 3). In a transverse section of the shoot base several such tracheid nests could be observed (Fig. 4). Some of these nests showed a distinct group of small spherical cells at their periphery (Fig. 3, at arrows). These cells apparently originated from the activity of the wound cambium surrounding the tracheid nest. In a longitudinal section of a nest, the presence of such a group of cells was distinct (Fig. 5). The tracheid nest in this section was in direct contact with the vascular system of the shoot.

The cells of the earliest recognisable root meristemoid were small and contained densely staining cytoplasm and large nuclei. These cells stained intensely with azure B, indicating high levels of DNA and RNA (Fig. 6). Similar studies have not frequently been undertaken. In French bean (*Phaseolus vulgaris* L.), which has no preformed initials, IBA at a concentration which readily enhanced rooting stimulated the rapid synthesis of proteins and subsequently of RNA (Kantharaj *et al.* 1979). Brossard (1977) found that during rooting in foliar discs of *Crepis capillaris* L. Wallr., root initiation was also accompanied by an increase in the starch content of the explants. A strongly starch-positive zone was always present in the parenchyma cells underlying the sites of root initiation. We also observed the occurrence of starch grains in cells close to the root initials (Fig. 5 at arrowheads), and a histochemical test for starch was always positive in the cells of the meristemoids. The accumulation of starch has long been associated with *in vitro* shoot formation (see Thorpe 1980), and may therefore be a general feature of *de novo* primordium initiation.

The sites of root primordia formation are shown to be associated with resin canal differentiation (Smith & Thorpe 1975; Montain *et al.* 1983a). However, in the present study the sites of root initiation were probably correlated more with the tracheid nests than with the resin canals. This is in agreement with data from several unrelated plant species, where formation of adventitious roots has been shown to take place in the vicinity of the differentiating vascular tissue of the organ from which the root arises (Esau 1965; Hartmann & Kester 1975; Brossard 1977). With further time in culture the root meristemoids located at the periphery of the tracheid nests began to differentiate into root primordia (Fig. 7). Further development of these primordia rapidly produced the normal configuration of the root apex. The periclinal divisions of the cells at the periphery of the primordia led to the differentiation of the root cap (Fig. 8).

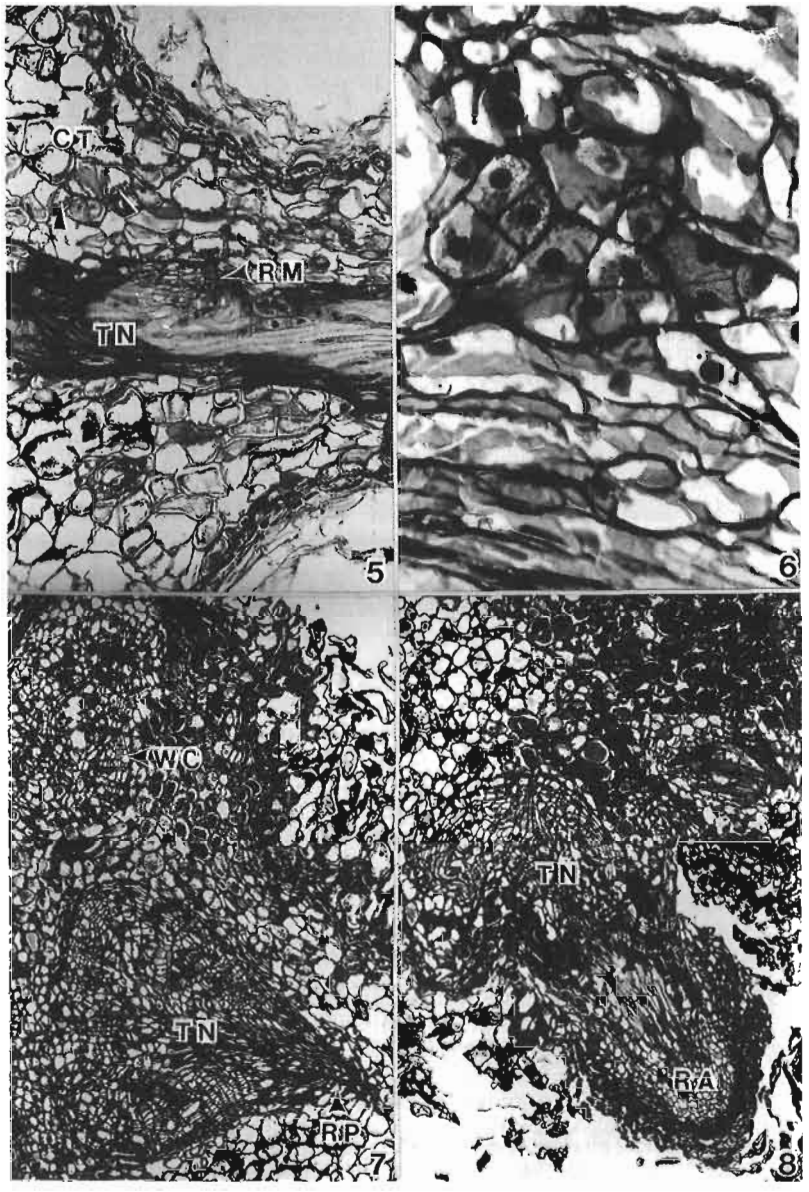

FIG. 5–8—Anatomy of root initiation in adventitious shoots of white spruce. Fig. 5: A longitudinal section of the shoot base showing a root meristemoid (RM) developing at the periphery of a tracheid nest (TN) (×195). Note accumulation of starch grains in some cortical cells (CT) located in the vicinity of a meristemoid. Fig. 6: Cells of the meristemoid containing densely staining cytoplasm and large nuclei (×1070). Fig. 7: A transverse section of the shoot base showing the wound cambium (WC) and the development of a root primordium (RP) at the periphery of a tracheid nest (TN) (×195). Fig. 8: Emergence of a root with a well-organised root apex from the shoot base (×195).

In a longitudinal section of a plantlet, the root is clearly seen to emerge near a tracheid nest which was in direct contact with the vascular system of the shoot (Fig. 9). The root was formed above the proliferating callus mass, and within the shoot base. Furthermore, the wound cambium, from which the roots arose, acted in some respects like the pericycle of an intact root. In black and white spruce the development of tracheid nests precedes and may be necessary for the development of a functional root system, as the roots were in vascular connection with the shoots through these nests (Fig. 10). Similarly, in *Pinus sylvestris* L. hypocotyl cuttings the tracheid nests were connected to the vascular system of the hypocotyl through short tracheids (Gronroos & von Arnold 1985). In both white (Fig. 11) and black (Fig. 12) spruce plantlets the continuity of the vascular system between the shoot and root was confirmed by clearing them with sodium hydroxide and histological staining.

In several conifer species, lack of a proper vascular connection between the root and the shoot has been considered to be a main reason for the high mortality of the tissue culture-derived plantlets upon transplanting (David 1982). The reason for this is that quite often *in vitro* rooting occurs within the basal callus. In the spruces, under study here, roots were formed above the callus, which probably accounts for their solid connection to the shoot vascular tissue. Development of a normal, healthy, and functional root system at a high frequency is a prerequisite for plantlet formation, if the technology is to be of commercial importance. Under the culture conditions used in the present study more than 80% rooting was achieved in both black and white spruce (Rumary & Thorpe 1984). This level of rooting is among the highest reported in conifers cultured *in vitro*. Finally, the root systems of both black and white spruce portrayed typical morphology which, along with the solid connection between the root and shoot, allowed for the production of plantlets with normally functioning root-shoot axes.

ACKNOWLEDGMENTS

This research was supported by NSERC of Canada Grants No. A6467, G0818, and G1438 to T. A. Thorpe.

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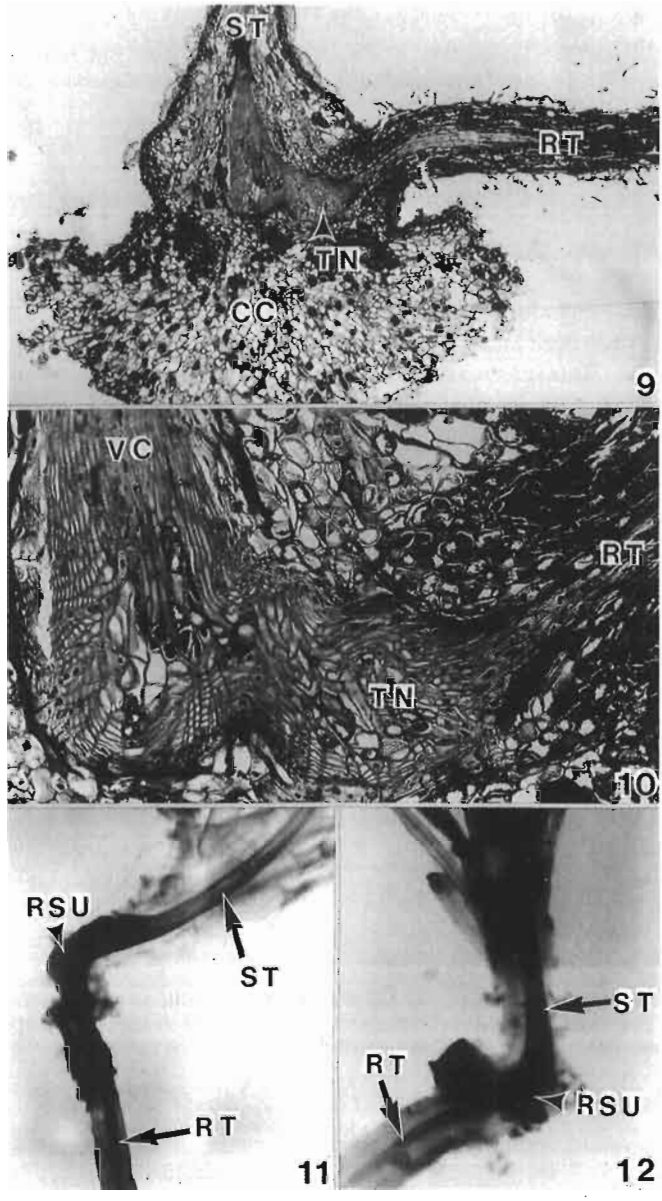

FIG. 9–12—Anatomy of the root-shoot union of the tissue culture-derived plantlets of white spruce. Fig. 9: A longitudinal section of the plantlet showing the basal part of the shoot (ST), callus cells (CC), tracheid nest (TN), and the root (RT) (×70). Note the root emerges from the tracheid nest located in the vicinity of the vascular cylinder of the shoot, and above the basal callus mass. Fig. 10: Magnified view of the root-shoot union showing the vascular connection of the root to the shoot through the tracheid nest (×208). Fig. 11 and 12: Plantlets of white spruce (Fig. 11) and black spruce (Fig. 12), showing continuity of the vascular tissue of the root (RT) and the shoot (ST) and the root-shoot union (RSU).

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