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Seed germination, seedling growth and haustorial induction in *Santalum album*, a semi-root parasite

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Abstract. Seed germination and early growth of the seedling in *Santalum album*, a semi-root parasite, is independent of the host but seedling establishment seems to be dependent on the establishment of host contact. About half of the seedlings, raised in aseptic cultures, showed drying of the shoot tip upon transfer to fresh medium and subsequent development of a large number of adventitious shoot buds on the hypocotyl. Attempts to induce rooting in these shoot buds were unsuccessful. Gum tragacanth, which induces haustoria in two Scrophulariaceous root parasites, and xenognosin, the active fraction from gum tragacanth, were effective in inducing haustoria in *S. album* in the absence of the host.

Keywords. *Santalum album*; sandal wood; semi-root parasite; seed germination; seedling establishment; adventitious shoot buds; haustorial induction.

1. Introduction

Santalum album (Santalaceae) is an arborescent semi-root parasite native in South India, but planted elsewhere in some tropical regions. It is the source of an essential oil, sandal wood oil. Although several studies have been carried out on the morphogenetic potentialities of embryo, endosperm and hypocotyl segments in aseptic cultures (Rangaswamy and Rao 1963; Rao 1965; Rao and Bapat 1978; Bapat and Rao 1979, 1984; Lakshmisita 1979; Lakshmisita *et al* 1979, 1980), experimental studies on seed germination and seedling establishment are very limited (Srimathi and Rao 1969; Nagaveni and Srimathi 1980, 1981). Such studies are useful not only in understanding the biology of the parasite, but also in its propagation through seeds, which is so far, the only method available for this species. We have investigated the requirement for seed germination, seedling growth and haustorial development and this paper reports the results.

2. Materials and methods

Two samples of dried fruits of *Santalum album* were obtained from Bangalore during September 1980 and October 1981 and stored in Delhi in screw cap bottles under laboratory conditions for about three months before use. The fruit is a drupe. The seed lacks a seed coat and contains a massive endosperm enclosing a well-developed embryo. The entire fruit as well as seeds after removing the fruit wall were used for germination.

Fruits were surface-sterilized with 7–10 % cetavlon (cetrimide concentrate, Imperial Chemical Industries) for 5 min followed by 10 min treatment with sodium hypochlorite (10 %, v/v). The fruits were then sown in petriplates lined with two layers of

Whatman No. 1 filter paper moistened with 9 ml of distilled water. In one experiment fruits were washed in running tap water for 1–6 days before surface sterilization and culture. In another experiment, the fruits were scarified by treating them with concentrated sulphuric acid for 5–25 min, washed in sterile water and then sown for germination.

To raise seed cultures, the fruit wall was manually removed. Subsequently they were surface-sterilized and sown for germination in the same way as described for intact fruits. As the seeds took over 14 days for germination, petriplate cultures usually got infected. Studies were, therefore, largely carried out in aseptic cultures for which Nitsch (1951) basal medium was used. In one experiment the effect of different carbohydrate sources (sucrose, glucose, xylose, mannose and galactose) and also casein hydrolysate, on seed germination and seedling growth was tested.

In petriplate cultures three replicates of 25 fruits/seeds each were raised for each treatment. In aseptic cultures, 24 fruits/seeds were used for each treatment and the experiment was replicated at least once. The cultures were maintained at 23 $\pm 2^{\circ}$C under diffused light conditions. One set of cultures was maintained in dark.

The effect of many growth regulators—gibberellic acid (GA_3), 6-benzylaminopurine (6-BAP), kinetin (Kn) and thiourea (TU) (added singly at concentrations ranging from 10^{-6} to 10^{-4}M) and ethrel (50–400 μg/ml) were studied on germination of cultured fruits and seeds.

The seedlings raised in aseptic cultures were transferred to fresh medium after 30–35 days of culture. On transfer, some of the seedlings showed drying of the shoot tip followed by development of shoot buds on the hypocotyl. To induce roots, the shoot buds were excised and transferred to fresh medium. Apart from Nitsch basal medium (1951), Murashige and Skoog's medium (1962), Tepfer's medium (Tepfer *et al* 1963), and White's medium (1963) supplemented with various hormones (indoleacetic acid (IAA), indolebutyric acid (IBA), naphthaleneacetic acid (NAA), 6-benzylaminopurine (6-BAP)) were also tried.

Aseptically grown seedlings were transferred to the soil. Some of the seedlings were transplanted to pots in which seedling of *Lantana camara* L., a natural host, were growing. The seedlings were maintained under laboratory/field conditions.

For induction of haustoria, the efficacy of gum tragacanth (an exudation product of a legume—*Astragalus* sp.) and 'xenognosin' (a host recognition factor isolated from gum tragacanth) which are effective in inducing haustoria, in two Scrophulariaceous root parasites, were tested.

Aqueous extract of gum tragacanth (Sigma) was prepared by boiling the required amount of the gum in double-distilled water. The resulting jelly-like solution was incorporated in culture tubes (final concentration of the gum tragacanth being 3·33 mg/ml and 1·66 mg/ml) before autoclaving.

A stock solution of xenognosin was prepared in methanol (0·8 mg in 40 ml) and stored at -4°C until use. The required amounts of the solution (to give final concentration of 10, 20 and 30 μg/ml) were filter-sterilized and loaded onto the sterilized filter paper discs (diameter 2 cm) under aseptic conditions with a sterilized syringe. Methanol was allowed to evaporate and the discs were placed (one each per culture tube) on the surface of the agar medium (0·6% agar). Two-three month old seedlings were transferred to this medium. The cultures were incubated under diffuse light at 23 $\pm 2^{\circ}$C.

Anatomical studies were carried out on the details of development of shoot buds on

the hypocotyl and of haustoria induced in the absence of host. The material was fixed in 10 % aqueous acrolein for 24 hr at 0°C and dehydrated through a 2-methoxy ethanol–ethanol–*n*-propanol–*n*-butanol series (Feder and O'Brien 1968). The material was then infiltrated and embedded in JB_4 resin (Polysciences USA). Sections (2 μm thickness) were stained with toluidine blue (Merck) (0·1 %) in 0·1 M acetate buffer, pH 4·5 (Feder and O'Brien 1968), mounted in DPX mountant (BDH) and observed.

3. Observations

3.1 *Fruit cultures*

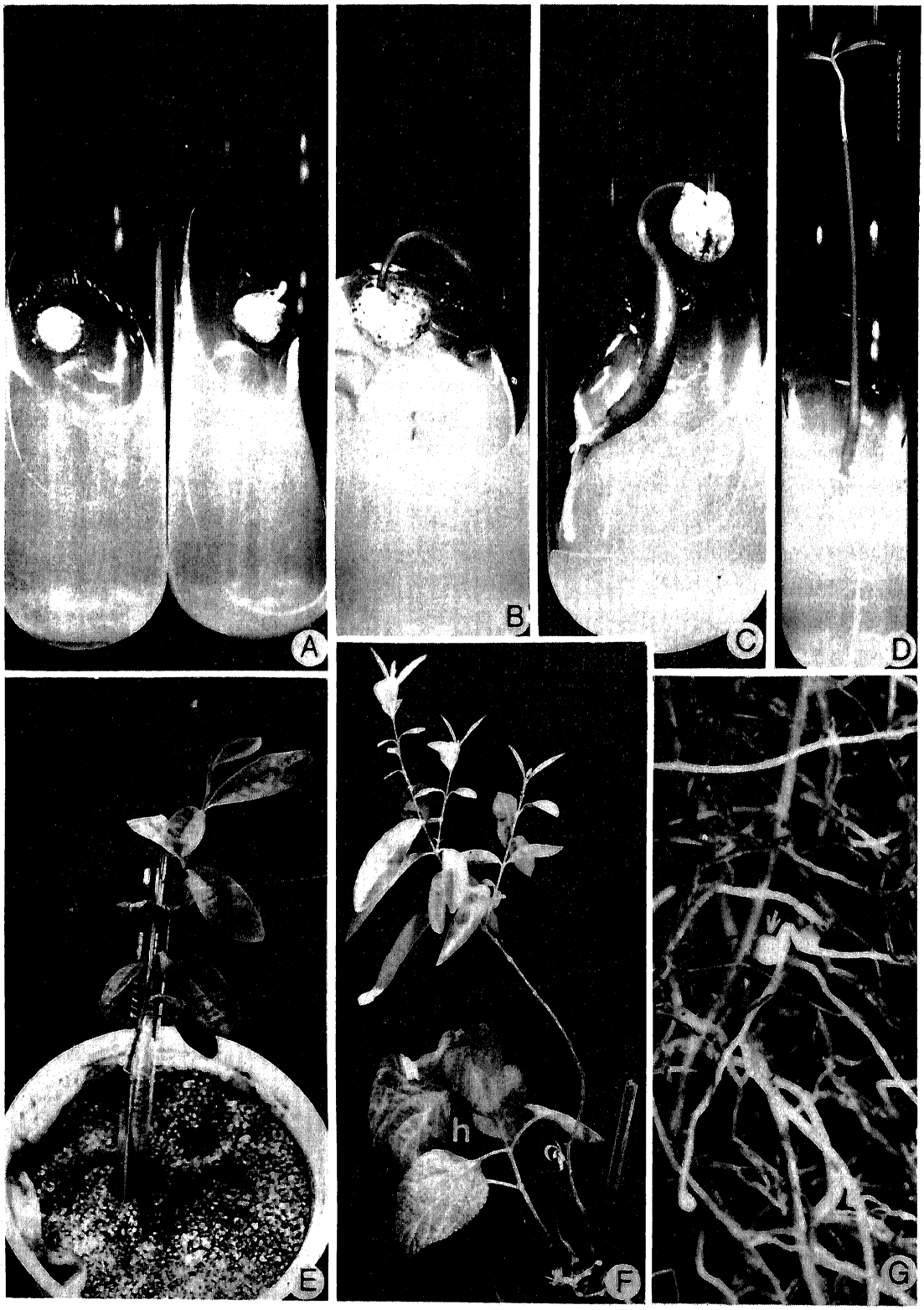
The fruits failed to germinate both in aseptic and petri plate cultures. Neither water washing nor scarification could induce germination. The scarified fruits showed hydration and splitting of the fruit wall after 20 days of incubation but no germination. Similarly treatment with chemicals such as GA_3, 6-BAP, Kn, TU and ethrel failed to induce germination under both light and dark conditions.

3.2 *Seed cultures*

Seeds readily germinated in petriplate as well as in aseptic cultures. There was no need for any pre-treatment. Agar (0·6 %) medium alone supported germination of up to about 40 % seeds. Addition of mineral salts, vitamins, and amino acids (basal medium, BM) and sucrose improved germination only up to about 50 %. Light was found essential for germination. Following hydration a small protuberance appeared on one side of the decoated seed in about 10 days. In another two days, the protuberance showed a splitting (figure 1A). Almost all the seeds showed these responses on all the germination media tested. In many of the seeds, however, germination did not proceed beyond this stage. In others, radicle emerged in about 14 days of culture and formed woolly root hairs near the tip. Subsequently the hypocotyl elongated with a prominent arching (figure 1B) while the cotyledons still remained enclosed within the endosperm. Eventually the hypocotyl became erect, lifting the cotyledons and the surrounding endosperm from the surface of the medium (figure 1C). Gradually the endosperm dried and the cotyledons unfolded. The shoot produced 3–5 pairs of leaves in about three months (figure 1D).

Amongst different carbohydrates (added singly to BM) tested, xylose, mannose, and galactose induced germination in only about 10 % of the cultures. Even in seeds that showed germination, further growth was very poor as cotyledons failed to emerge in most of them. Sucrose and glucose supported good germination (*ca* 50 %) and seedling growth.

Addition of casein hydrolysate (200–500 mg/l) to BM did not improve germination and seedling growth. Although IAA (1·5 mg/l) induced germination in about 60 % seeds, further growth was very poor; cotyledons remained enclosed by the endosperm even after 60 days of culture. The root showed elongation as well as branching. 2,4-dichlorophenoxyacetic acid (1–3 mg/l) was strongly inhibitory. It induced germination only in about 5 % of the seeds. Although the seeds showed swelling and irregular splitting, radicle failed to emerge in most of the seeds. Even in seeds showing emergence of radicle, cotyledons failed to emerge.



Figures 1A–G. Seed germination and seedling growth. **A.** Seed cultures showing protuberance and splitting. **B.** 23-day-old culture showing elongation of hypocotyl with prominent arching. **C.** Hypocotyl has become erect lifting cotyledons enclosed by endosperm. **D.** Forty-day-old seedling. Cotyledons have abscised. **E.** A seedling in sterilized vermiculite two months after transfer. **F.** *Santalum* seedling together with a seedling of the host (h), *Lantana camara*, one year after transplantation. **G.** Portions of roots of seedlings shown in **F** to show haustorial contact (arrow) between the roots of parasite and of host.

3.2a *Transplantation of seedlings*: Three-month-old seedlings grown on basal medium were transferred onto the fresh medium in 250 ml flasks. The seedlings continued growth and reached up to the cotton plug in about one month. The seedlings were then transferred to plastic pots containing sterilized vermiculite (figure 1E) and maintained in the culture room under diffuse light for one month. They were irrigated with distilled water or Hoagland and Arnon's (1950) nutrient solution. The growth in distilled water was as good as in Hoagland and Arnon's solution. Subsequently, they were transferred to autoclaved garden soil in pots and maintained in the culture room. The seedlings survived in pots in the culture room for up to about eight months. They could not be grown under laboratory conditions as they started drying in about two weeks after shifting.

Some of the seedlings from vermiculite were transferred to garden soil in pots, together with a seedling of *Lantana camara* and maintained in the laboratory. These seedlings survived the transfer and (figure 1F), produced many new branches. One of them which was subsequently kept under field conditions (in shade) continued growth. When plants were carefully uprooted and washed, after about 15 months, *Santalum* roots showed a large number of haustorial connections with the host roots (figure 1G).

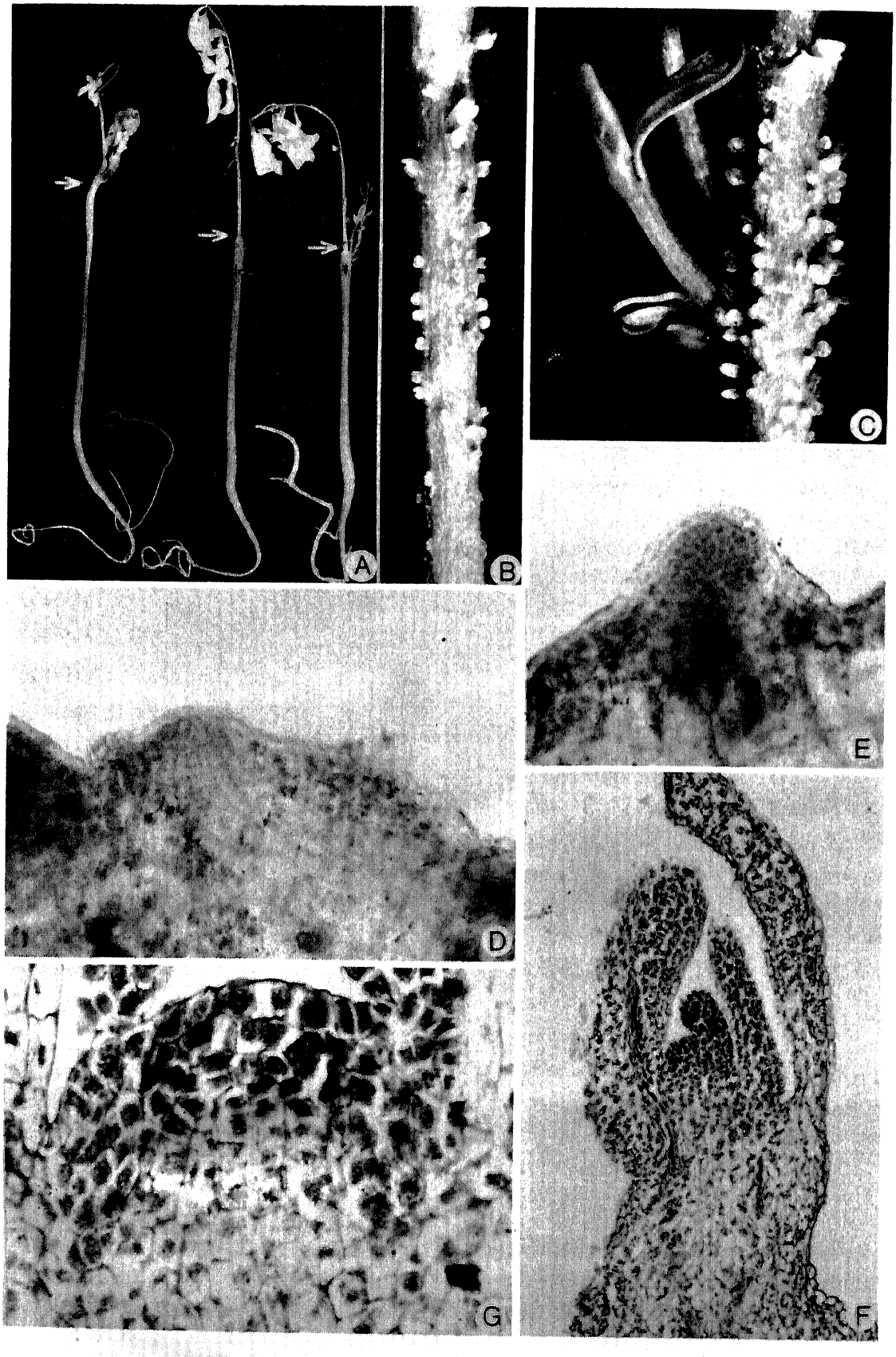
3.2b *Origin of adventitious shoot buds*: On transferring to fresh medium, the shoot in about 60 % of the seedlings ceased growth and the shoot tip started drying. The drying extended towards the base up to the level of the cotyledons (which generally abscise by the time 3–4 pairs of leaves develop) (figure 2A). Such seedlings showed development of many protuberances on the surface of the hypocotyl just below the dried shoot tip in 10–15 days of transfer. Subsequently, these protuberances developed into normal shoot buds (figure 2B, C). Such shoot buds developed only upon transfer of the seedlings and never in the original cultures maintained for many months without transfer.

The shoot buds originated through the activity of subepidermal cortical cells. These cells became meristematic, and produced a compact meristemoid (figure 2D). This resulted in the development of protuberance on the epidermal surface (figure 2E). Shoot apex soon differentiated at the tip of the meristemoid facing the epidermis. Shoot primordia emerged by rupturing the epidermal layer.

Anatomically the shoot buds had normal shoot apex (figure 2F, G). Initially vascular strands of the shoot bud were not connected to the vascular bundles of the axis. On subsequent growth these vascular strands established contact with those of the main axis. Out of the large number of shoot buds that were initiated earlier, only 2–4 of them developed into branches (figure 2C), while rest of them did not grow further.

All attempts to induce rooting in the shoot buds by manipulating the concentrations of auxins and cytokinins of the medium were unsuccessful.

3.2c *Induction of haustoria*: Aseptically grown three-month-old seedlings were transferred to the medium supplemented with gum tragacanth/xenognosin. On media containing gum tragacanth (1·66 and 3·33 mg/ml), although the lateral roots which originated after the transfer showed swellings at the tips in about a month, they did not develop haustoria. However on subsequent transfer to pots filled with vermiculite, many haustoria developed on the roots of these seedlings in about two months. Some of them developed into autohaustoria by establishing contact with the neighbouring roots of the same seedling (figure 3A). Anatomy of the fully developed haustoria showed a typical structure reported for Santalaceous members (Kuijt 1977; Rao 1942).


A
B
C
D
E
F
G

The sections showed the mantle, the collapsed layer and the central body (figure 3B).

Xenognosin was also effective in haustorial induction. In 20% of the cultures the roots became swollen only at the highest concentration (30 μg/ml) of xenognosin tested. These swellings were similar to those formed on the gum tragacanth-supplemented media. When such seedlings were transferred to pots containing vermiculite, many haustoria as well as autohaustorial connections developed in about four weeks (figure 3C).

4. Discussion

Using aseptic culture, Rangaswamy and Rao (1963) reported germination of seeds only in the presence of casein hydrolysate or coconut milk and suggested that the seeds may require some stimulus from the host roots. Subsequently, Srimathi and Rao (1969), however, obtained germination of seeds both in petriplate cultures and in soil without any pretreatment or addition of any growth substances. Germination of the entire fruit was, however, not satisfactory.

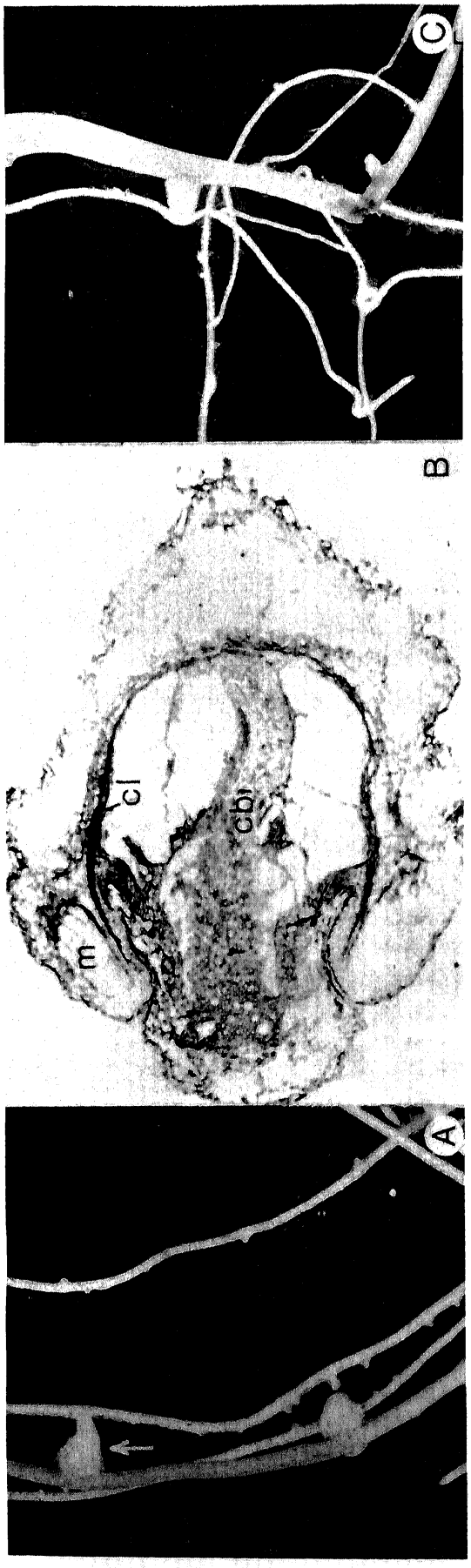
In the present investigation also entire fruits failed to germinate whereas seeds germinated readily without any pretreatment or addition of any stimulants. Apart from acting as a barrier for hydration, the fruit wall also seems to contain some inhibitors. Scarification of fruits with concentrated H_2SO_4, although allowed hydration of seeds, failed to promote germination.

Seeds germinated readily without any pretreatment and produced normal seedlings. Light was essential for germination. Unlike many other root parasites, in *Santalum album* it appears that seed germination and early seedling growth are independent of the host stimulus. It requires neither a carbohydrate source nor any exogenous growth substance. The seeds contain a massive endosperm filled with food material to sustain seedling growth until the development of leaves. Seedling growth could be maintained for many months without providing a carbohydrate source in the medium.

When the aseptically grown seedlings were transferred to soil, they could not survive for more than a few months. However, when transferred in the vicinity of a host, the seedling readily established haustorial contact with the host and continued growth. Thus for a successful establishment of the seedling in soil, development of haustorial contact with host root seems to be necessary.

An intriguing observation in the present study is the drying up of the shoots in about 50% of the seedlings upon transfer to fresh medium, and subsequent development of a large number of adventitious shoot buds. The development of adventitious buds depended on drying of the shoot and drying never occurred unless the seedling was transferred. These responses were not affected by the composition of the medium. The death of the shoot did not affect the establishment of the seedling, as one or a few of the adventitious shoot buds grew and gave rise to a normal shoot. The factor(s) responsible for the death of the shoot and subsequent shoot bud development are not clear.

Figures 2A–G. Development of adventitious shoot buds. **A.** Some representative seedlings photographed to show drying of shoot tip and development of shoot buds from the hypocotyl. Arrows indicate position of abscised cotyledons. **B, C.** Enlarged portions of hypocotyl showing a large number of shoot buds. In **C** some of the shoot buds have grown further to form new shoots. **D–G.** Anatomical details of shoot bud development. **D, E.** Differentiation of subepidermal meristemoid. **F.** LS shoot bud. **G.** LS shoot apex.



Figures 3A–C. Haustorial induction. **A.** Gum tragacanth-induced autohaustoria. **B.** Off median LS of autohaustoria passing through one of the roots showing mantle (m), collapsed layer (cl) and central body (cb). **C.** Xenognosin-induced autohaustoria.

Because of the importance of the sandalwood tree in essential oil industry, many attempts have been made to obtain clonal multiplication through tissue culture (Rao and Bapat 1978; Bapat and Rao 1979; Lakshmisita *et al* 1979, 1980). Hypocotyl segments, shoot segments and shoot tips have been used, and callusing and differentiation of embryoids as well as shoot buds have been achieved in these explants. Plantlets have been readily obtained from embryoids. However, induction of roots in the shoot buds has not been very successful. Rao and Bapat (1978) reported rooting in a few cultures on a medium containing NAA and IBA. In the present investigation, rooting could not be induced in shoot buds although as many as 85 combinations of different growth substances including those used by Rao and Bapat (1978) were tried. Induction of rooting in these shoot buds, would greatly facilitate clonal propagation. As there is no callusing in the production of shoot buds, the feasibility of obtaining true clones through this method is more than that through embryoids.

The parasitic behaviour of *Santalum* has been described by Barber (1907), Pilger (1935) and Rao (1942). Until recently there were no experimental studies on haustorial induction in any root parasitic taxa. Studies during the last few years (Nickrent *et al* 1979; Riopel and Musselman 1979; Lynn *et al* 1981; Sahai and Shivanna 1981, 1984) have shown that gum tragacanth is effective in inducing haustoria in *Agalinis purpurea* and *Sopubia delphinifolia* both of the Scrophulariaceae. Subsequently the active fraction from gum tragacanth was isolated and characterized, and was named 'xenognosin' (Lynn *et al* 1981).

Both gum tragacanth and xenognosin were effective in inducing haustoria in *Santalum album* also. Haustorial inducing factor in *S. album*, therefore, appears to be the same or very much similar to that effective in *Agalinis purpurea* and *S. delphinifolia*. Induction of haustoria in the absence of host would facilitate experimental studies on the details of host recognition and haustorial differentiation.

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