Growth of *Oncobasidium theobromae* Talbot & Keane in Dual Culture with Callus Tissue of *Theobroma cacao* L.

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**INTRODUCTION**

It is now established that the tulasnelloid basidiomycete *Oncobasidium theobromae* (Talbot & Keane, 1971) is the pathogen causing the vascular-streak dieback disease of cocoa in Papua New Guinea. There is a wide range of resistance in the cocoa population and this has been exploited for disease control. However, development of a rapid standard technique for resistance screening requires a regular supply of basidiospores, the only known propagules. Under natural conditions these are only produced during periods of very wet weather. The spores germinate on the surface of young leaves and penetrate the veins. They also germinate freely on agar media and produce germ tubes up to 400 μm long, but further development has not been observed away from the host. The fungus appears to flourish only in association with the living host; it invariably dies as soon as the host dies and it has never been observed to grow saprophytically on dead host wood.

Live callus tissue cultures can provide a useful growth medium for fungi whose nutritional requirements are unknown; a number of obligate fungal parasites have been grown on callus tissues of their hosts (Ingram, 1973). This paper reports the establishment of callus tissue of *Theobroma cacao* and the maintenance and subculture of callus infected with *O. theobromae*.

**METHODS**

The basic medium used for establishing and maintaining callus tissue cultures was that devised by Murashige & Skoog (1962). The modifications of Hall & Collin (1975) were also studied. The medium was made up in distilled water/filtered green coconut milk (2:1, v/v). Stock solutions of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin were made up in 50% (v/v) ethanol and dilute hydrochloric acid respectively, and kept at 2 °C. These hormone substitutes were added to the basal medium at 10⁻⁶ M. The medium was autoclaved in 100 ml flasks or McCartney bottles at 121 °C for 2 min. Difco agar or Oxoid agar no. 3 were used at 0.8% (w/v). The pH of the medium was not adjusted; after autoclaving it was 4.5.

The cocoa clones were of the Trinitario type, selected at Keravat, Papua New Guinea, for superior agronomic features and maintained for breeding purposes and distribution to growers. Clones KA2 101 and KA 105 are resistant to vascular-streak dieback in the field; KA5 104, K5 and K6 are susceptible.

Callus was obtained from both somatic anther tissue and from ungerminated embryos. Unopened flower buds were surface-sterilized for 10 s in 96% (v/v) ethanol; whole anthers plus filaments were dissected out aseptically and placed on the surface of the medium in McCartney bottles. To obtain embryos, the mucilage and testa were removed from ripe cocoa beans of clone K5 and the cotyledons were severed close to the cylindrical embryo,
which was then surface-sterilized for 10 min in 7% (w/v) filtered calcium hypochlorite solution and the radicle and plumule were removed. The cylinder of tissue remaining, which was approximately 5 mm long, was planted radicle end downwards in the medium, so that half the cylinder was below the agar surface. The light regime during callus formation did not appear to be important, but the explants were never exposed to bright light. The cultures were maintained at 25 °C. Callus and dual cultures were kept in diffuse daylight and exposed to intermittent fluorescent laboratory light for 12 h every day. Subcultures were made at approximately 4-weekly intervals.

Mycelium of *O. theobromae* was obtained from infected stems. Bark was removed from 5 cm lengths of stem and these were surface-sterilized, as above, for 10 min. A section, 1 cm long, was removed from each end and the remaining stem was placed on water agar containing streptomycin (100 units ml⁻¹). Three to four days later, mycelium had grown on to the agar. Callus tissue was inoculated by placing 9 mm discs of mycelium in contact with the callus.

**RESULTS AND DISCUSSION**

**Growth of cocoa callus**

When anther filaments were placed on agar medium callus tissue formed at the cut ends. There was sufficient tissue for subculturing after 2 weeks. No callus formation occurred from pollen, and only rarely from somatic tissue in the anther itself. Bacterial contamination was never a serious problem. Callus formed more readily from larger anther filaments (2 mm long) than from smaller ones. The four clones used, K6, KA2 101, KA5 104 and K24 105, did not differ in their ability to produce callus from filaments, and the calluses were morphologically indistinguishable.

Embryo sections produced callus from the exposed cambial tissue submerged in the agar. Within 2 weeks the callus had proliferated and lifted the original explant clear of the agar. Clones were established from four embryos of clone K5.

Callus was initiated most readily from both anther filaments and embryos when the medium was supplemented with green coconut milk, 2,4-D and kinetin, but initiation also occurred in the absence of the two hormone substitutes. Continued growth on basal medium supplemented with coconut milk only was very slow, callus taking 1 to 2 months to double in size. Growth of such callus was very even and after 3 months the surface had a smooth white appearance. On the same medium supplemented with 2,4-D and kinetin, callus grew much faster, doubling in size every 2 weeks. Growth was irregular, the callus was friable and many of the cells became brown and stopped growing. After about 4 weeks, callus growing on basal medium supplemented with coconut milk, 2,4-D and kinetin grew more slowly and its appearance became similar to that grown on basal medium plus coconut milk only. Callus behaved similarly on the medium of Hall & Collin (1975).

Callus growth rates varied between clones. Callus isolated from clone KA5 104 grew rapidly in the first two subcultures but growth then declined. The reverse was true for clones KA2 101 and K6, which grew more rapidly after two transfers. After 6 months and eight transfers, growth of all the clones had declined to less than 25% of their maximum rate.

**Growth of Oncobasidium theobromae**

Mycelium of *O. theobromae* grew from the ends of surface-sterilized infected stems on to water agar and formed colonies up to 4 cm in diameter. The colonies reached their maximum size in 4 to 7 days, and during this period growth ceased if the stem was removed. Keane, Flentje & Lamb (1972) reported that subcultures grew slowly on sweet potato-sucrose agar
and produced monilioid hyphae in older cultures, but this was not confirmed during the present investigations. Subcultures from colonies formed from infected stem sections grew very poorly and erratically on standard media. Quite vigorous initial growth was occasionally obtained on the medium of Murashige & Skoog (1962) amended with $10^{-8}$ M-indole acetic acid and kinetin. However, many replicates failed to grow, even on a single batch of medium or from a single piece of infected stem. None of the successful isolates survived a second subculturing. The addition of green coconut milk to standard media did not improve growth, and the fungus would not grow on membrane-filtered expressed xylem sap, with or without the addition of various antioxidants such as phenylthiourea and glutathione.

Mycelium of *O. theobromae* grew vigorously on callus tissue and grew out on to the agar medium, eventually forming a dense yellowish mycelium over both the callus and the medium. The callus continued to grow even when completely covered by the fungus and apparently viable callus was recovered after incubation for 8 weeks. When pieces of agar containing growing mycelium of *O. theobromae* were removed from dual cultures and placed on identical medium in the absence of callus tissue, the fungus made no further growth. Isolates from the Keravat area did not show any obvious morphological variation; however, only a few isolates have been examined. Although callus was established from clones showing a wide range of resistance to vascular-streak dieback disease, there was no consistent difference in fungal growth and behaviour in dual culture.

Dual cultures were subcultured approximately every 4 weeks. The dual system remained stable for six transfers, but in many cultures one or other organism eventually became dominant. When the callus senesced, mycelial growth was very vigorous, the dying callus providing an excellent substrate for growth of the fungus. This was also noticeable on freshly transferred cultures, where the fungus grew out from damaged areas of callus. Microscopic examination of infected callus showed that mycelial growth was intercellular; no cellular penetration was seen, even in dying tissue. The fungus appears to utilize nutrients which leak from the callus cells, and although at present the nutritional requirements for growth of *O. theobromae* are unknown, growth on callus tissue is a considerable advance towards their identification.

In some well-established dual cultures which were at least 4 weeks old, the fungus formed areas of swollen short hyphae on the callus and on the surrounding agar. They appeared identical to the monilioid hyphae described by Talbot & Keane (1971). Basidia, which are produced from monilioid hyphae under natural conditions, were not seen in dual culture. However, the production of monilioid hyphae under laboratory conditions suggests that this stage in basidial formation is not mediated by specific temperature or light regimes since the cultures were subjected to constant temperature and irregular light. Under natural conditions the change from vegetative to monilioid hyphae occurs on the bark surface after the mycelium has grown out of freshly exposed leaf scars. Since this change also occurs in older dual cultures it seems likely that the stimulus is either nutrient deprivation or an accumulation of metabolic products. Environmental stimuli, particularly temperature and certain wavelengths of light, may be necessary to induce basidial formation in these monilioid hypha.

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Short communication

REFERENCES


