Neth. J. Pl. Path. 99 (1993) 227–230 SHORT COMMUNICATION

A novel method for producing basidiocarps of the cocoa pathogen Crinipellis perniciosa using a bran-vermiculite medium

G.W. GRIFFITH* and J.N. HEDGER

Department of Biological Sciences, University College of Wales, Penglais, Aberystwyth, Dyfed, SY23 3DA, Wales

Accepted 1 June 1993

Abstract

A novel method for the production of basidiocarps from the mycelia of *Crinipellis perniciosa* is described. This involved the colonization of a bran-vermiculite medium with pure culture of the fungus, prior to application of a peat-based casing. Basidiocarp production was induced by hanging the cultures in a cabinet where they were subjected to a daily cycle of wetting and drying. The method was successfully and reproducibly used to fruit isolates of all four known biotypes of the fungus within 10–16 weeks of inoculation.

Additional keywords: Witches' broom disease, tropical basidiomycete, basidiocarp production.

The agaric *Crinipellis perniciosa* (Stahel)Sing. is the causal agent of witches' broom disease (WBD) of cocoa (*Theobroma cacao* L.). Since basidiospores are the only infective propagules of *C. perniciosa* (Evans and Bastos, 1980), a regular supply of basidiocarps is necessary for investigation of the processes of pathogenesis. Most of the early work on WBD was carried out in areas where the disease was rife, so basidiocarps were readily available. However, in recent years a great deal of work on WBD has been conducted in laboratories in Europe and North America leading to the development of several methods for the fruiting of *C. perniciosa* under artificial conditions.

Using naturally infected cocoa meristems (brooms) placed in a 'broom cabinet' to simulate tropical conditions, Suarez (1977) and Rocha and Wheeler (1982, 1985) investigated the climatic conditions necessary for basidiocarp production. Spraying the brooms with water for 8 h per day, a temperature of 25 °C and moderate levels of light in a 12 h photoperiod induced optimal basidiocarp production. This method requires a supply of brooms and provides basidiospores of uncertain origin (since the cocoa tissues were infected in the field), but large numbers of basidiocarps can be produced over the course of several months.

The earliest successful attempt to fruit *C. perniciosa* artificially was by the German pathologist Stahel (1919) who hung a mat of mycelium in a porcelain pot under a tree in Surinam. Purdy et al. (1983) and Purdy and Dickstein (1990) have refined this method with some success by subjecting aseptically grown mycelial mats, hung on sterile cocoa brooms or other supports, to simulate tropical conditions as above.

* Present address: School of Biological Sciences, Memorial Building, University College of North Wales, Bangor, Gwynedd LL57 2UW, Wales.

Merchan (1979), Purdy et al. (1983) and Pickering and Hedger (1987) have also obtained basidiocarps by inoculating sterile cocoa brooms embedded in water agar (in conical flasks) with plugs of mycelium. Most recently Bastos and Andebrhan (1987) have reported the production of basidiospores directly on callus-like stromata in mycelial cultures under ambient (i.e. Brazilian laboratory) conditions.

All these methods, with the exception of that of Bastos and Andebrhan (1987), have been used with limited success in our laboratory for several years but the minimum time between inoculation and basidiocarp production was at least 3 months. In addition, mycelial mats were susceptible to contamination by other microbes.

In the present study, a method combining the fluctuating moisture conditions required for the fruiting of *C. perniciosa* with more conventional methods of mushroom cultivation (Elliott, 1985) has been developed. By this method basidiocarps were produced more rapidly, in greater numbers and over a longer period of time than by those previously described.

A solid bran-based medium was prepared containing 40 g vermiculite, 50 g bran (Bran Flakes; Weetabix Ltd.), 6 g $CaSO_4$. $2H_2O$, 1.5 g $CaCO_3$ (both BDH Ltd.) and 120 ml distilled water (moisture content 65–70%, pH 7.0–7.5). The ingredients were mixed well, dispensed into round aluminium foil dishes (11 cm diameter, 2.5 cm deep; Alcan Ltd; 30 g per dish), wrapped in foil and autoclaved (15 min, 15 psi). When cooled, the bran medium was inoculated with three 5 mm agar discs (cut with a cork borer) of mycelium (from 1 to 3-week-old cultures grown on 3% malt-0.5% yeast extract agar) and incubated at 25 °C in a plastic container (a seed propagator; 80 x 30 x 20 cm), containing wet paper towels to prevent desiccation of the medium.

When the mycelium had covered the surface of the bran medium (usually 3–4 weeks), the cultures were covered with a casing layer about 5 mm thick (5–10 g per culture), made of 200 g coarse peat, 50 g $CaCO_3$, 50 g vermiculite and 125 ml distilled water (moisture content 70–75%, pH 7.0–7.5). These ingredients were mixed well and pasteurised at 80 °C for 15 min before use. The cultures were then incubated for 3–4 weeks as before.

At the end of the incubation period, the foil covering was discarded, loose casing was gently rubbed away and the cultures placed or hung vertically in a broom cabinet (Purdy and Dickstein, 1990) at 27 °C. A Defensor 500 humidifier was set to spray deionized water from 0200 to 0800 and 1600 to 1700 (7 h per day in total) with 12 h of light (0800 to 2000; fluorescent warm white 65–80W tubes).

A total of 18 different isolates were successfully fruited by this method, including isolates of all 4 known biotypes of *C. perniciosa*, namely the C-biotype (cocoa), L-biotype (liana; Fig. 1), S-biotype (*Solanum* species) and B-biotype (*Bixa orellana*) from several countries (Griffith, 1989). Basidiocarps were produced from over 70% of cultures after 2–12 weeks incubation in the broom cabinet. More than 40 basidiocarps were produced in some cases, the average being about 15 per culture.

The dynamics of basidiocarp production was similar to that observed in *Agaricus bisporus* (Flegg and Wood, 1985). In the case of the L-biotype isolate, SCL2, 10 to 30 basidiocarps were produced over a 2–3 day period, after the cultures had been in the broom cabinet for 4 to 6 weeks. One to three progressively smaller flushes (2–10 basidiocarps) were subsequently observed at 1–3 week intervals.

Basidiocarps were morphologically similar to those observed in the field (Hedger et al., 1987). Basidiocarps of L-biotype isolates were larger (15–35 mm diameter) and crimson in colour, compared to the smaller (8–20 mm diameter), paler fruit bodies produced by isolates of other biotypes. In contrast, basidiocarps produced by the mycelial mat method are usually smaller (4–6 mm diameter) than those which occur in nature (Purdy and Dickstein, 1990), possibly because of the smaller amount of substrate available to the

Fig. 1. Basidiocarps of the L-biotype of *Crinipellis perniciosa* (isolate SCL2 from Amazonian Ecuador) obtained by the bran-vermiculite method after 24 days incubation in a broom cabinet. Scale bar = 1 cm.

underlying mycelium. Basidiospore production, as judged by the deposition of spore prints on agar media, and viability (98–100%) were comparable to that observed in basidiocarps produced from cocoa brooms (Brownlee et al., 1990).

Some isolates produced basidiocarps more rapidly than others. For instance, the two *Solanum* biotype isolates fruited most rapidly (after 15–20 days in the broom cabinet), whereas most isolates of other biotypes produced the first flush of basidiocarps after 4–6 weeks. It is likely that the mycelial growth rates of isolates is an important factor in basidiocarp production using this method, since complete colonization of the bran medium prior to casing was found to be a prerequisite to fruiting. Of the four isolates which were not successfully fruited, three were found to be very slow-growing on agar media (Griffith, 1989). It was also observed that the mycelium on the surface of the casing layer of the most productive cultures developed a crimson pigment when placed in the broom cabinet. Similar observations of mycelial mats were made by Purdy et al. (1983).

This method provides a convenient and rapid method for producing basidiocarps of *C. perniciosa*. It has proven useful for studies of the breeding biology and biotrophic growth of this important pathogen without reliance on imported cocoa brooms (Griffith, 1989). Its reliability compared to other methods may be due to the fact that the fungal mycelium is to some extent buffered from extreme moisture levels by the comparatively large volume of the medium.

The success of preliminary experiments, in which cultures were watered daily with a watering can, suggests, that the method may be simplified further. It may also prove useful for fruiting other small agarics adapted to growth in conditions of fluctuating moisture levels. For example, *Marasmiellus troyanus* (Murr.)Dennis, a species occupying a similar niche to the L-biotype of *C. perniciosa*, was found to fruit well using this method (Griffith, 1989).

Acknowledgements

We thank Mrs Carol Parry for technical assistance. The financial support of the UK Science and Engineering Research Council to G.W.G. is gratefully acknowledged.

References

Bastos, C.N. & Andebrhan, T., 1987. *In vitro* production of basidiospores of *Crinipellis perniciosa* (Stahel)Sing., the causative agent of witches' broom disease of cocoa (*Theobroma cacao* L.). Transactions of the British Mycological Society 88: 404–409.

Brownlee, H.E., Hedger, J.N. & Scott, I.M., 1990. Host extracts cause morphological variation in germ-tubes of the cocoa pathogen, *Crinipellis perniciosa*. Mycological Research 94: 543–547.

Elliott, T.J., 1985. Spawn-making and Spawns. In: P.B. Flegg, D.M. Spencer & D.A. Wood (Eds), The biology and technology of the cultivated mushrooms. John Wiley and Sons, London, U.K., p. 131–140.

Evans, H.C. & Bastos, C.N., 1980. Basidiospore germination as a means of assessing resistance to *Crinipellis perniciosa* (witches' broom disease) in cocoa cultivars. Transactions of the British Mycological Society 74: 525–536.

Flegg, P.B. & Wood, D.A., 1985. Growth and Fruiting. In: P.B. Flegg, D.M. Spencer & D.A. Wood (Eds), The Biology and technology of the cultivated mushroom. John Wiley and Sons. London, U.K., p. 141–177.

Griffith, G.W., 1989. Population structure of the cocoa pathogen *Crinipellis perniciosa* (Stahel) Sing. Ph.D. thesis, University of Wales.

Hedger, J.N., Pickering, V. & Aragundi, J.A., 1987. Variability of populations of the witches' broom disease of cocoa (*Crinipellis perniciosa*). Transactions of the British Mycological Society 88: 533–546.

Merchan, V.M., 1979. Formación en medios de cultivo de basidiocarpos de *Crinipellis perniciosa*. Ascolfi Informa (Colombia) 5: 54–56.

Pickering, V. & Hedger, J.N., 1987. Production of basidiocarps of the witches' broom pathogen *Crinipellis perniciosa* in *in vitro* culture. Transactions of the British Mycological Society 88: 404–406.

Purdy, L.H. & Dickstein, E.R., 1990. Basidiocarp development on mycelial mats of *Crinipellis perniciosa*. Plant Disease 74: 493–496.

Purdy, L.H., Trese, A.T. & Aragundi, J.A., 1983. Proof of pathogenicity of *Crinipellis perniciosa* to *Theobroma cacao* by using basidiospores produced in *in vitro* cultures. Revista Theobroma (Brazil) 13: 157–163.

Rocha, H.M. & Wheeler, B.E.J., 1982. Water balance as an important factor in basidiocarp production by *Crinipellis perniciosa*, the causal fungus of cocoa witches' broom. Proceedings of the Eighth International Cocoa Research Conference, 1981, Cartagena, Colombia. Cocoa Producers' Alliance, Lagos, Nigeria. p. 381–368.

Rocha, H.M. & Wheeler, B.E.J., 1985. Factors influencing the production of basidiocarps and the deposition and germination of basidiospores of *Crinipellis perniciosa*, the causal agent of witches' broom disease on cocoa (*Theobroma cacao* L.). Plant Pathology 34: 319–328.

Stahel, G., 1919. Contribution to the knowledge of Witchbroom disease. Tropical Agriculture, Trinidad 9: 167–176.

Suarez, C., 1977. Growth of *Crinipellis perniciosa* (Stahel)Singer *in vivo* and *in vitro*. Ph.D. thesis, University of London.