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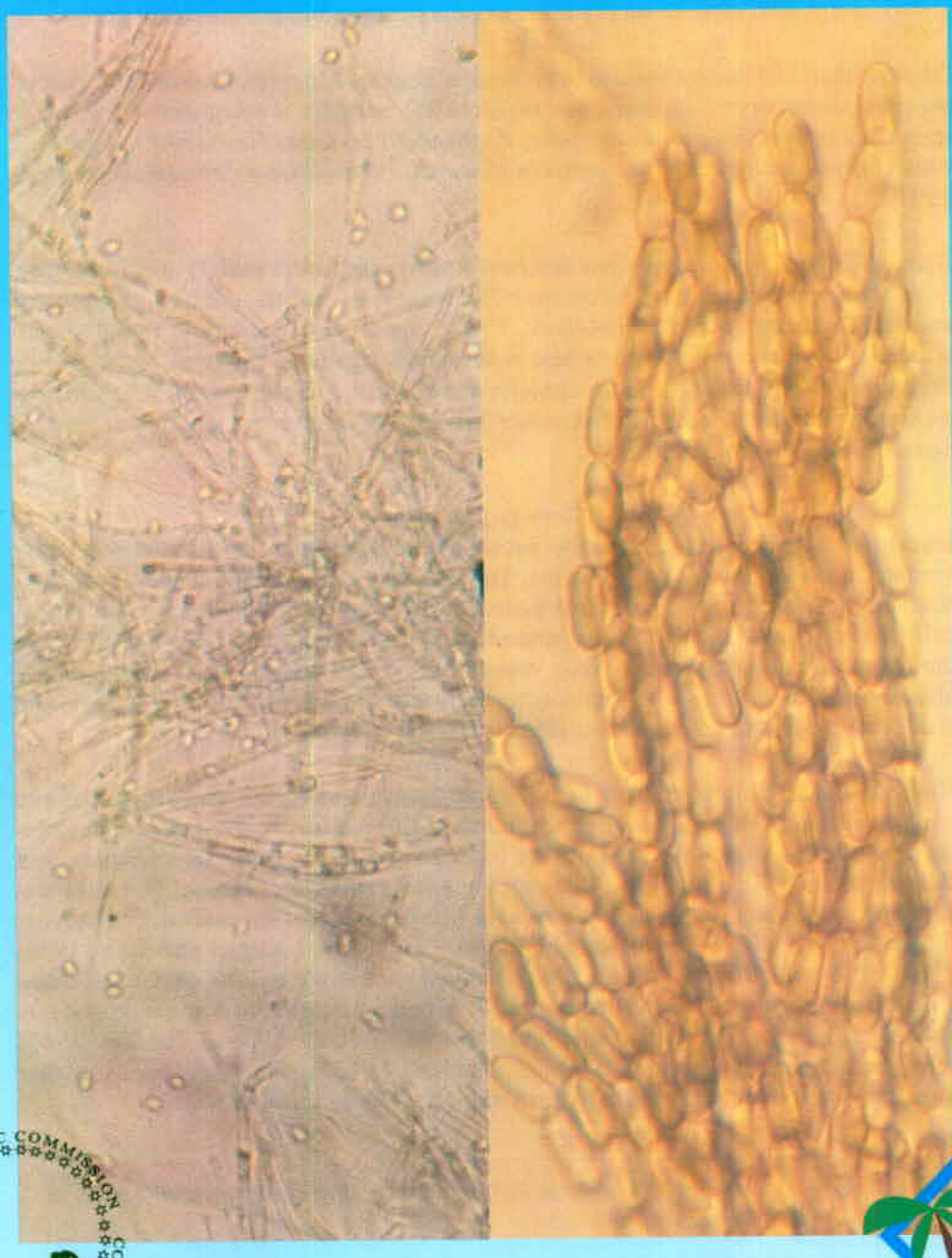
TECHNIQUES AND MEDIA FOR ISOLATION, CULTURE, STORAGE AND BIOASSAY OF METARHIZIUM ANISOPLIAE AND BEAUVERIA BRONGNIARTII



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PRAP

Fungi have adapted to a wide variety of habitats and a wide range of diets: they can be predacious (feeding on protozoa and nematodes), parasitic or saprophytic. Entomopathogenic fungi often cause spectacular epizootics in insects; they accomplish infection through the integument and generally are easy to mass produce and safe for mammals and thus good candidates for biological control. Their major disadvantage is the often low effectiveness in controlling the insect population. This can possibly be overcome by screening for an isolate with superior (field) characteristics (virulence, sporulation, persistence). The most effective isolate often derives from the target species or a closely related species (Soares *et al.*, 1983; Poprawski *et al.*, 1985) so it is advisable to survey these insects for possible effective isolates.

Of more than 700 known species of entomopathogenic fungi (from about 100 genera, mostly Deuteromycotina and Entomophtorales) only six are registered for use in insect control: *Ashersonia aleyrodis*, *Beauveria bassiana*, *Hirsutella thompsonii*, *Metarhizium anisopliae*, *Paecilomyces lilacinus*, *Verticillium lecanii* (Roberts *et al.*, 1991).

Beauveria and *Metarhizium* are the best known and most widely used of these. *Beauveria* spp. has been found on over 500 species of insects, mainly coleopterans and lepidopterans (Hall and Papierok, 1982): *Beauveria bassiana* is found worldwide and has been used more or less successfully against a range of insects while *Beauveria brongniartii* is more specific for scarabs. (Glare, 1992). *Metarhizium anisopliae* has been described from over 200 species of insects including at least 70 Scarabaeidae (Latch, 1965).

PATHOGENESIS

Insect pathogenic fungi can usually penetrate the integument and sometimes enter via the digestive tract or the spiracles. The infective unit (usually a spore) attaches itself to the cuticle, germinates and penetrates by degrading the integument physically and/or enzymatically. After penetration, yeast-like hyphal bodies multiply in the haemocoel. They produce toxic metabolites which kill the host. The mycelial phase then grows throughout the insect's body and finally penetrates the cuticle and produces infective units on the outside of the host (Photos 1 and 2).

High virulence, a high number of conidia produced on cadavers, good dispersal and long persistence under unfavourable conditions (including the absence of hosts) determine the effectiveness of fungi as biocontrol agents. Both *Metarhizium* and *Beauveria* have these qualities. They produce large quantities of conidia on the cadaver. *Metarhizium anisopliae* can remain infective in soil for well over one year (Latch and Falloon, 1976; Milner and Lutton, 1976; Muller-Kogler and Stein, 1976; Rath, 1992) and Mikuni *et al.* (1982) showed that some strains of *Metarhizium anisopliae* can grow saprophytically in soil. Although *Beauveria* conidia do not survive as long in soil (Muller-Kogler and Stein, 1976), Gotwald and Tedders (1983) consider *B. bassiana* superior to *M. anisopliae* because of higher pathogenicity, higher spore production and the ability to grow saprophytically through soil.

Cover photo: Round spores of Beauveria (left) and oval spores of Metarhizium (right) at 100x magnification.

Photos 1 & 2. Taro beetle (Papuana uninodis) larvae and adults covered with sporulating mycelium of M. anisopliae (green, above) and B. brongniartii (white, below).

HOST RANGE, SAFETY

Fungi are potential control agents for many insects. Some fungal species are rather host specific (e.g. *Aschersonia aleyrodis* infects only whiteflies), others like *M. anisopliae* have a very wide host range as a species but each *M. anisopliae* isolate has a limited range. Mammals are generally not susceptible to insect pathogenic fungi but laboratory tests on relevant non-target hosts (e.g. honey bees) are recommended for registration (Hall *et al.*, 1982).

Care should be taken in interpreting these lab data. Susceptibility under laboratory conditions does not imply infections under field conditions: the potential host and fungus may not frequent the same environment under natural circumstances and even if they do frequent the same environment, biotic factors (antagonists and host related factors) or abiotic factors (UV-light, moisture, temperature, pH, aeration, inorganic matter, pesticides) in the environment of the host insect may hinder the development of the fungus.

ISOLATION AND PRODUCTION

Metarhizium and *Beauveria* can be isolated from soil or insects with selective media (Appendix 3).

For initial and small-scale multiplication the fungi can be grown in petri dishes (Appendix 1) on simple media like Molisch's agar, Emerson YpSs agar or Sabouraud's dextrose agar with yeast (SDAY) (Appendix 4). Antibiotics can be added to prevent contamination from bacteria.

Liquid cultures (Appendix 5) are usually a step to (small) mass culture. To grow the fungi in liquid culture, spores derived from petri dish cultures are mixed with culture medium in a flask and left to grow on a shaker for several days. The volume of medium is kept small compared to the flask size (e.g. 50 ml in 250 ml flask), a plug that allows some air exchange is used and the medium is continuously shaken so sufficient air is available for the growing fungus.

The most common procedure for mass production is to grow the fungus on organic materials (rice, bran) until sporulation and to introduce the spores (with or without the growth medium) in the field (Appendix 6). The hyphae that have developed after 3 to 4 days in a liquid culture or alternatively in petri dish cultures can be used to inoculate sterilized rice. Production can be about 10^9 *Metarhizium* spores/g rice, or about five times more *Beauveria* spores (Photos 3 and 4). The dried rice/spore mix can be used as it is, if desired the rice can be crushed or the spores can be shaken or rinsed from the substrate.

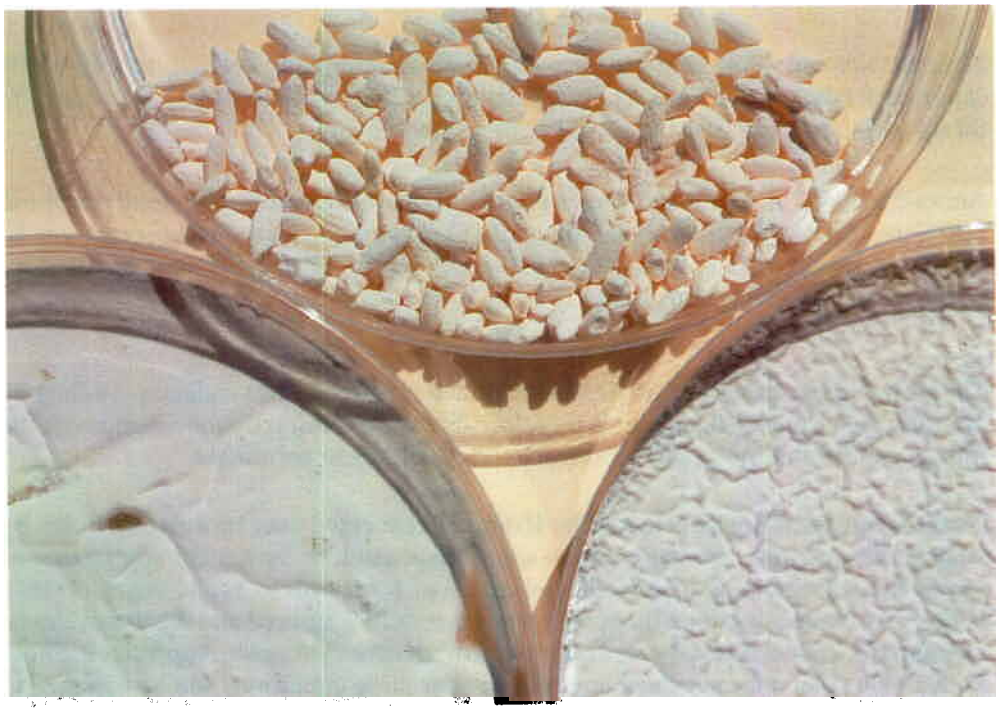

Photo 3. Sporulating mycelium of the fungus Beauveria on agar medium and rice.

Photo 4. M. anisopliae cultured on sterilized rice in an autoclavable bag.

Storage

Metarhizium and *Beauveria* can be maintained in storage by subculturing in screw cap bottles (slants, Appendix 1) or in petri dishes. One sample can be stored at 4°C for up to 4–6 months, another at –20°C for up to 4–5 years.

Another method is to store the spores only (without the mycelium) in dried condition at 4°C using anhydrous silica gel (Appendix 7). The advantages are that spores can be stored for long periods, mites cannot survive these dry conditions, and repeated inocula can be taken from one bottle.

Laboratory assays

The ability to penetrate the cuticle is a major determinant of the virulence of a fungus. To study the virulence the insect is therefore directly or indirectly inoculated with the fungus and kept in moist surroundings to allow spores to germinate.

Direct inoculation can be achieved by letting insects crawl over sporulating fungi, by dusting with conidia, spraying with a spore suspension or by topical application of spores in a solution (e.g. by topical application of 10^6 spores in 3–5 µl vegetable oil). In this way contact between the spores and the cuticle is assured and the insect can be exposed to a known dose (spores are counted in a haemocytometer, Appendix 2, Photo 5). A fungus that does not show any effect under these conditions can be assumed to have no effect in natural circumstances.

Indirect inoculation can be done by bringing spores into the (natural) environment of the insect, e.g. for soil insects, by mixing spores with soil. The insect is exposed to a given concentration (e.g. 10^7/g soil). Usually the spores are cultured on organic medium which is either included or the spores are separated.

Mortality and fungus infection are recorded, e.g. twice weekly. Since the purpose of the tests usually is to examine the effect of the initial concentration of the fungus, the dead insects are removed to prevent extra contamination. Cadavers can be placed in a petri dish lined with moist tissue paper to promote fungal growth.

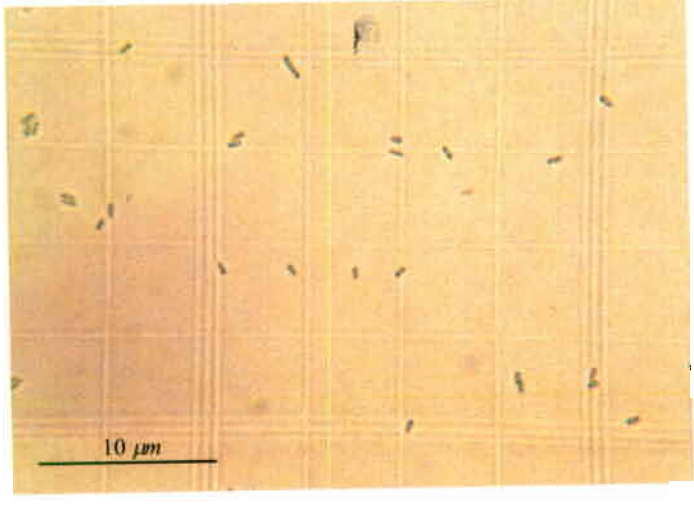

Photo 5. M. anisopliae spores in a haemocytometer, 40x magnification. Bar = 0.01 mm = 10 µm.

APPENDIX

1. Petri dish, slants

Usually, disposable sterile 90 mm petri dish are used. After autoclaving the medium is left to cool to 50–60°C before adding antibiotics or pouring. A magnetic stirrer can be included during autoclaving to facilitate mixing in antibiotics and to make sure the solution stays properly mixed while cooling. One litre of solution is sufficient for 30–40 petri dishes.

The procedure for slants is identical. The medium is poured into sterile (packed sterile or autoclaved), wide mouthed, screw cap tubes. The tubes are inclined while the medium is congealing so a slope is formed which provides a relatively large surface for the culture in a small container.

Spores are then spread over the surface of the medium in the petri dish or screw cap bottle. After a week to 10 days the culture has usually sporulated and is ready for storage or harvesting.

2. Haemocytometer

A haemocytometer consists of a microscope slide with a central area containing a fine grid which becomes visible under the microscope. The grid consists of squares with a known surface and, if covered by a coverslip, a known height so the volume enclosed can be calculated. Usually the surface is 1/400 mm^2 and chamber height 0.1 mm, so each square/cube represents a volume of (1/400 x 0.1 mm^3 =) 25 x 10^{-5} mm^3.

A small drop of the well mixed spore suspension is placed on the grid and covered with a coverslip. If the distribution of the particles is not even, the procedure should be repeated. Under a magnification of 200x–1000x the number of particles per square is calculated by counting e.g. 50–100 squares. The number of particles per ml is calculated by multiplying the average for one square by a factor, in the above example, 4 x 10^6.

The number arrived at is an estimate. The precision of the estimate will depend on the sample and the number of squares counted: a higher dilution will make easier but less accurate counting and counting few squares will be faster but again less accurate. Example:

10 g of a sporulated fungal growth on rice is suspended in 100 ml water with 0.05% Tween 80, mixed well and examined with the haemocytometer.
The suspension contains too many particles to make a proper count and it is diluted 1/10.
The count for the 1/10 suspension: 240 spores in 80 squares = 3 spores/square
per ml: 3 x 4 x 10^6 = 1.2 x 10^7spores/ml.
The concentration of the original suspension is 10x higher = 1.2 x 10^8spores/ml.
The number of spores/gram of culture is 10x higher (for every gram 10 ml of water was used) = 1.2 x 10^9 spores/gram.

3. Selective media

For *Metarhizium* (and *Beauveria*)
Milner's agar (Liu *et al.*, 1993)

Neutralized soya peptone	10 g
Dextrose	10 g
Bacteriological agar	20 g
Chloramphenicol (in acetone)	1 g
Cycloheximide	0.5 g
Distilled water	1 litre
Dodine	1 g/l

Autoclave at 121°C, 15 minutes

Specifically for *B. bassiana* (Doberski, J.W. and Tribe, H.T., 1980)

Glucose	40 g
Neopeptone (or Difco Proteose peptone no.3)	10 g
Agar	15 g
Crystal violet	0.01 g
Cycloheximide	0.25 g
Distilled water	1 litre
Chloramphenicol (in acetone)	0.5 g

Autoclave at 121°C, 15'

4. Culture media

(Autoclave 15 minutes 115°C)

Molisch's agar

Peptone	10 g
Sucrose	20 g
K_2PO_4	0.25 g
$MgSO_4$	0.25 g
Agar	15 g

+ 1 litre water

Emerson YpSs agar

(ready made from Difco, 40.5 g/l or)

Yeast extract	4 g
Starch	15 g
K_2PO_4	1 g
$MgSO_4$	0.5 g
Agar	20g

+ 1 litre water

Sabouraud's dextrose agar with yeast (SDAY)

Yeast extract	2 g
Polypeptone peptone	10 g
Dextrose or saccharose	20 g
Agar	15 g

+ 1 litre water

Antibiotics

Penicillin + streptomycin (300–500 u/ml) have to be added after autoclaving when the temperature is <60°C

or

chloramphenicol (0.51 mg/ml = 0.5–1 g/litre) is heat resistant and can be added before autoclaving dissolved in acetone.

5. Liquid culture media (after Barnes *et al.*, 1975)

For *Metarhizium anisopliae* (or *Beauveria*)

per litre	
D-glucose	10 g
Yeast extract	10 g
Tween 80	0.05%

For *Beauveria bassiana*
per litre

D-glucose	10 g
Casitone or peptone	10 g
Tween 80	0.05%

50 ml aliquots in 250 ml bottles
Plug with cotton cork and cover with aluminum foil
Autoclave 20', 121°C, leave to cool
Add some spores from petri dish culture (1/8 petri dish)
Grow on shaker at room temperature for 3–4 days
In lieu of the cotton cork the flask can be covered with aluminum foil for autoclaving and after adding the spores the flask can be covered with sterile tissue paper

Antibiotics: as above.

6. Mass culture on rice
500 g rice in autoclavable bag, autoclave 2 h, 115–121°C
Add 150 ml tap water, autoclave 20 minutes, 121°C
Cool down and brake up clumps
Add 1 flask of liquid culture or about 10^9 spores harvested from a petri dish (semi-sterile)
Close the bag, mix well, spread open (in the closed bag), leave as much air inside as possible before closing
Store at 25–30°C with some daylight coming in (no direct sunlight)
After 3–5 days the mycelium should be growing properly, break the clumps again
Check regularly, break up clumps and discard if contaminated
After about 1 week the first sporulation often starts
After about two weeks the culture is ready
Open the bag and let the culture dry for 3–5 days (turn daily)
To count: rinse 10–20 g (add 10 ml water-Tween 80/g of sample), count in a haemocytometer (Appendix 2)

7. Storage on silicagel:
Method of choice in IMI (Smith, 1991):
1. Fill heat resistant screw cap bottle with 6–22 mesh non-indicating silicagel. Sterilize by dry heat (180°C, 3 h)
2. Place in tray with water, freeze (–20°C)
3. Prepare spore suspension in cooled 5% (w/v) skimmed milk
4. Add the suspension to the silicagel to 3/4 wet it, mix thoroughly
5. Store for 10–14 days at 25°C until dry (caps loose)
6. Store at 4°C in air tight containers (add indicator gel to absorb moisture)
7. Retrieve by scattering some crystals on medium.

References

Barnes G.L.; Boethel D.J.; Eikenbary R.D.; Criswell J.T.; Gentry C.R. (1975) Growth and sporulation of *Metarhizium anisopliae* and *Beauveria bassiana* on media containing various peptone sources. *Journal of invertebrate pathology*, **25**, 301–305.

Doberski J.W.; Tribe H.T. (1980) Isolation of entomogenous fungi from elm bark and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisopliae*. *Transactions of the British Mycological Society*, **74**(1), 95–100.

Glare T.R. (1992) Fungal pests of scarabs. *In*: Jackson T.A.; Glare T.R. (*eds.*) Use of pathogens in scarab pest management. pp 63–77.

Gotwald T.R.; Tedders W.L. (1983) Suppression of pecan weevil (Coleoptera: Curculionidae) populations with entomopathogenic fungi. *Environmental entomology*, **12**, 471–474.

Hall I.M.; Papierok B. (1982) Fungi as biological control agents of arthropod pests. *Parasitology*, **84**, 205–240.

Hall R.A.; Zimmerman G.; Vey A. (1982) Guidelines for the registration of entomopathogenic fungi as insecticides. *Entomophaga*, **27**, 121– .

Latch G.C.M.; Falloon R.E. (1976) Studies on the use of *Metarhizium anisopliae* to control *Oryctes rhinoceros*. *Entomophaga*, **21**(1), 39–48.

Liu Z.Y.; Milner R.J.; McRae C.F.; Lutton G.G. (1993) The use of dodine in selective media for the isolation of *Metarhizium* spp. from soil. *Journal of invertebrate pathology*, **62**, 248–251.

Mikuni T.; Kawkami K.; Nakayama M. (1982) Survival of the entomogenous fungus *Metarhizium anisopliae*, causing muscardine disease of the silkworm, *Bombyx mori*, in soil of mulberry plantations. *Journal of sericultural science of Japan*, **51**, 325–331.

Milner R.J.; Lutton G.G. (1976) *Metarhizium anisopliae*: Survival of conidia in the soil. Proceedings of the 1st International Colloquium on Invertebrate Pathology, 428–429.

Muller-Kogler E.; Stein W. (1976) Gewachshausversuche mit *Metarhizium anisopliae* (Metsch) Sorok. zur infektion von *Sitona lineatus* L. im Boden. *Pflanzenschutzberichte*, **83**, 96–108.

Poprawski T.J.; Marchal M.; Robert P.H. (1985) Comparative susceptibility of *Othiorynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomophomycetes. *Environmental entomology*, **14**, 247–253.

Rath A.C. (1992) *Metarhizium anisopliae* for control of the Tasmananian pasture scarab *Adoryphorus couloni*. *In*: Jackson T.A.; Glare T.R. (*eds.*) Use of pathogens in scarab pest management. pp 217–228.

Roberts D.W.; Fuxa J.R.; Gaugler R.; Goettel M.; Jacques R.; Maddox J. (1991) Use of pathogens in insect control. *In*: Pimentel D.; Hanson A.A. (*eds.*) CRC Handbook of pest management in agriculture.

Smith D. (1991) Maintenance of filamentous fungi. *In*: Maintenance of microorganisms. London: Academic. pp 133–159.

Soares G.G. Jr.; Marchal M.; Ferron P. (1983) Susceptibility of *Othiorhynchus sulcatus* (Coleoptera, Curculionidae) larvae to *Metarhizium anisopliae* and *M. flavoviride* (Deuteromycetes, Hyphomycetes) at two different temperatures. *Environmental entomology*, **12**, 1886–1891.

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