

(CONVENTION. By one or more persons and/or a Company.)

631221

Form 4.

COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

(1) Here insert (in full) Name of Applicant or Applicants, followed by Address(es).

X (1) INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (I.N.R.A.)
We of 147 rue de l'Universite, 75341 Paris Cedex 07, France

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled: (2)
MICROORGANISM-BASED PESTICIDE COMPOSITIONS, THEIR PRODUCTION PROCESS
AND THEIR APPLICATION IN AGRONOMY

(3) Here insert number(s) of basic application(s).

which is described in the accompanying complete specification. This applications is a Covention application and is based on the application numbered: (3)
89 08553

(4) Here insert Name of basic Country or Countries, and basic date or dates.

for a patent or similar protection made in: (4) France
on 27th June 1989

My
Our address for service is WATERMARK PATENT & TRADEMARK ATTORNEYS
290 Burwood Road, Hawthorn, Victoria, Australia.

DATED this27th..... day ofJune.....19.....90..

(5) Signature(s) of Applicant(s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

5) INSTITUT NATIONAL DE LA RECHERCHE
AGRONOMIQUE (I.N.R.A.)
by [Signature]
D. B. Mischlewski
Registered Patent Attorney

To: THE COMMISSIONER OF PATENTS.

WATERMARK PATENT & TRADEMARK ATTORNEYS

(CONVENTION. Company.)

Form 8

COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969

DECLARATION IN SUPPORT OF A CONVENTION
APPLICATION FOR A PATENT OR PATENT OF ADDITION(1) Here
insert (in
full) Name of
Company.In support of the Convention Application made by⁽¹⁾.....
INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA)

(hereinafter referred to as the applicant) for a Patent

(2) Here
insert title
of Invention.for an invention entitled:⁽²⁾.....MICROORGANISM-BASED PESTICIDE COMPOSITIONS, THEIR PRODUCTION PROCESS
AND THEIR APPLICATION IN AGRONOMY(3) Here
insert full Name
and Address,
of Company
official
authorized
to make
declaration.

I, ODILE FUCHS, of

I, ~~XXX~~ INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA)
of 147 rue de l'Université, 75341 PARIS CEDEX 07/FRANCE

do solemnly and sincerely declare as follows:

1. I am authorised by the applicant for the patent
to make this declaration on its behalf.

2. The basic application as defined by Section 141 of the Act was

(4) Here
insert basic
Country or
Countries
followed by
date or dates
and basic
Applicant or
Applicants.made in⁽⁴⁾ FRANCEon the 27th day of JUNE 1989, by INSTITUT
NATIONAL DE LA RECHERCHE AGRONOMIQUE (INRA)

on the day of 19xxxxx by

(5) Here
insert (in
full) Name
and Address
of Actual
Inventor or
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de la Libération, 21240 TALANT, France- 4. DESGRANGES Catherine, 40 bis rue
Alphonse Legros, 21000 DIJON, France-is/are the actual inventor s of the invention and the facts upon which the applicant
is entitled to make the application are as follow:The applicant is the assignee of RIBA Guy, GOUSSARD Jacqueline,
DURAND Alain and DESGRANGES Catherine4. The basic application referred to in paragraph 2 of this Declaration
was.....the first application made in a Convention country in
respect of the invention the subject of the application.

DECLARED at PARIS France

this 2nd day of July 1990

Pour le Chef du Service Odile FUCHS
Juridique et du ContentieuxMINISTRE DE L'AGRICULTURE
MINISTRE DE L'ÉDUCATION NATIONALE
Ministère chargé de la Protection et de
l'Environnement

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MICROORGANISM-BASED PESTICIDE COMPOSITIONS, THEIR PRODUCTION PROCESS AND THEIR APPLICATION IN AGRONOMY

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(56) Prior Art Documents
AU 66143/86 A01N 63/02 63/04
AU 50728/90 A01N 63/04 A01H 15/00

(57) Claim

1. A plant protection composition containing particles formed from at least one solid support carrying at least one microorganism effective against insect pests or crop diseases or for soil fertilization, said particles being colonized by the microorganism, the latter having an aptitude to sporulate under spreading conditions.

2. A composition as claimed in Claim 1, wherein it can be spread in a field immediately after it is obtained by direct growth and solid state fermentation on said particles.

3. A composition as claimed in either of Claims 1 and 2, wherein the microorganism is a mushroom.

16. Process for preparing a product to combat insect pests or plant diseases or for soil fertilization, which comprises direct growth by solid state fermentation of at least one microorganism on at least one solid support in particles, insoluble in the fermentation medium and the recuperation of said particles which are colonized by the microorganism, maturing of the growth being controlled to allow the microorganism to sporulate under distribution conditions.

631221

Form 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

Class

Int. Class

Application Number:

Lodged:

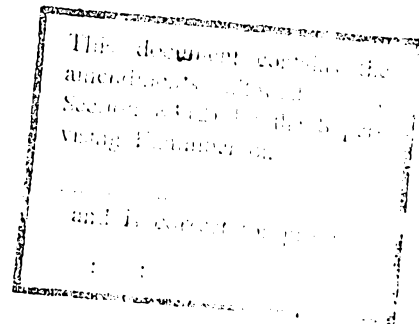
Complete Specification Lodged:

Accepted:

Published:

Priority:

Related Art:



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LOCKED BAG NO. 5, HAWTHORN, VICTORIA 3122, AUSTRALIA

Complete Specification for the invention entitled:

MICROORGANISM-BASED PESTICIDE COMPOSITIONS, THEIR PRODUCTION PROCESS
AND THEIR APPLICATION IN AGRICULTURE

The following statement is a full description of this invention, including the best method of performing it known to us

MICROORGANISM-BASED PESTICIDE COMPOSITIONS,
THEIR PRODUCTION PROCESS AND THEIR APPLICATION
IN AGRONOMY.

5

The present invention relates to new compositions with a base of entomopathogenic mushrooms, their production process and their application in agronomy.

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Numerous synthesized chemical compounds with relatively selective insecticide properties are already known, and although they are highly efficacious, their use is preferably limited, whether for economic or environmental reasons.

15

With previous methods it has been noted that appropriately selected microorganisms, notably certain mushrooms, could make an enormous contribution to an infected agrosystem and play the same role as chemical pesticides by limiting the development of crop insect pests and retarding multiplication of weeds or other mushroom plant pests.

20

Biological insecticides are more specific than the majority of chemical pesticides and therefore do not lead to secondary phenomena ; furthermore, they are non-toxic

to either invertebrates and vertebrates or humans. On this basis their use would be totally advantageous. Nevertheless, the following problems are encountered : first, the proliferation of entomopathogenic mushrooms is limited to warm and humid regions, whereas in temperate regions the fight against insects is not considered economically viable, given the high doses of mushrooms used ; secondly, in order to compensate for these drawbacks, a technology has been proposed, namely the use of solid supports, which are already known to professionals, as they are used in the production of compositions containing chemical insecticides or pesticides. GE 1002977 describes several compositions containing vermiculite as a solid support, each also containing a given pesticide, as well as various agents such as wetting agents.

US 4530834 relates to a production process for dried mycelium, comprising a certain number of steps, including a step for cultivation in a medium defined by the inventors as 'any commercially available medium', a step for treatment by a protective chemical agent for increasing viability of the mycelium, and a step for incubation and drying. It is specified that the products to be used for control of insects are ground and formed into wettable powder, then sprayed onto plants.

US 3013 946 also relates to a production process

for a mushroom composition having an insecticide effect, by cultivation of microorganisms of the Moniliales family on a solid vermiculite support. It is specified that fermentation is carried out over a period of 14 to 21 days, but without control of the state of the mushroom, and especially of the presence of spores. The culture medium is dried after fermentation before being used for spreading.

Mites are very well known for causing insect damage to crops, such as corn mites of the pyralide family *Ostrinia nubilalis* and *Ostrinia furnacalis*. Several species of entomopathogenic mushrooms are vulnerable to attack by mites ; thus the eggs of this insect are very sensitive to stocks of *Metarhizium anisopliae*, while the pupae die easily from pink pebrine with *Paecilomyces fumosoroseus*. Another mushroom, *Beauveria bassiana*, is responsible for white pebrine which attacks the nitid larvae and exists in an endemic state in populations of *Ostrinia nubilalis*. As with the majority of hyphomycetes, *Beauveria bassiana* generates two types of infectious propagules : blastospores by budding of mycelium, ensuing vegetative multiplication of the mushroom exclusively present in submerged culture, and conidia present in submerged culture and in solid state culture.

FR 2.394.606 relates to a production process for microorganisms, including *Beauveria bassiana* which can be

used in the fight against plant diseases, comprising mixing of an aqueous microorganism preparation with a clay suspension, which can be montmorillonite or bentonite.

5 A 1979 article by Ignoffo et al. (Journal of Economic Entomology, 72, 562-565) describes the production of *Beauveria bassiana* by fermentation in liquid medium, followed by drying and mixing with talc or other inert material, such as pearlite, kaolin, bentonite or starch.

10 EP-A-O 226 394 describes a direct fermentation process in a single step for various microorganisms on particles, more specifically vermiculite particles. The support thus treated can be used to inoculate cultures. In a characteristic realization the process comprises first mixing vermiculite, flour and water, sterilizing this mixture, then inoculating it with a microorganism such as a mushroom, storing it to mature and finally drying it to produce a particular product which can be used for treatment of crops.

20 The process described in EP-A-O 226 394 makes no mention of controlling the microorganism culture, whereas this parameter is essential for allowing efficacy of the product under the conditions for spreading and for optimizing manufacture. This patent does not describe a product in which the microorganism has an aptitude for sporulation

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in spreading conditions. Moreover, it provides sterilization of the support. In the illustrative examples given in EP A-O 226 394 it is noted that significant growth times are specified : 45 days in Example 1, 2 to 4 weeks in Ex-

5 amples 2 and 3. For the technician such time periods imply a lack of quality control and quantity control of the mushrooms in the final product. This process is also carried out in containers, the use of which produces growth of minimal thickness without any monitoring. The preferred support, such as illustrated in the examples of the prevoius patent, is vermiculite which proves unsuited to mechanized agro-

10 nomic use.

Therefore, the process described in EP-A-O 266 394

15 does not come up with a product able to sporulate in spreading conditions, under industrial growth conditions.

FR-A-2 298 951 relates to products in granulose form obtained by extrusion, which serve as inoculant, fertilizer or in other similar applications. Microorganisms are

20 added to a solid support and the mixture is treated until it reaches the desired solid form. The microorganisms, essentially of the Trichoderma type, are incubated on crushed barley corns or other grains, in closed containers.

25 This process disallows control of the quantity of biomass and of its quality.

FR-A-2 298 951 makes no mention of the use of particles or preplanted granules.

5 All these solutions to the problem of the biological fight against a crop insect pest, using mushrooms as insecticide, have the same disadvantages, that is, none of them is particularly efficacious in inhibiting propagation or growth of said insects.

10 The aim of the present invention is to obtain a product which offers high efficiency, stability, low production costs, due in particular to greatly reduced growth times and considerable ease of use.

15 A more particular object of the invention is a granular product, precolonized by microorganisms, whose maturing is controlled and which finds application in agronomic treatment.

20 The object of the present invention therefore is a product for agronomic processing with a base of at least one microorganism useful in the fight against insect pests and crop diseases or for soil fertilization, wherein it comprises particles formed from at least one solid support
25 carrying the microorganism, said particles being colonized by the microorganism, the latter capable of sporulating

under distribution conditions.

Said microorganism may be a bacteria permitting soil fertilization, such as Rhizobium, or an octomycorrhizian mushroom such as Laccaria. It may also be an entomopathogenic mushroom, effective against one or more crop insect pests, such as ground insects, especially scarabaeoidea and curculionides, stinging insects, especially cercopides, or boring insects, especially mites.

Said microorganism may also be a pathogenic mushroom vis-a-vis other mushrooms, such as Fusarium, non-pathogenic vis-a-vis plants but pathogenic vis-a-vis noxious Fusarium, or a Trichoderma pathogenic vis-a-vis Sclerotinia, Rhizotonia, Armillaria or Botrytis.

Said microorganism may also have a pathogenic effect on nematodes : it may thus be an Arthrobotrys.

A product containing one of these microorganisms especially has the advantage, compared to pesticides or customarily used fertilizers, of not polluting the environment and thus preserving flora and fauna.

According to a particular embodiment of the invention the product can be spread in fields directly

after it is obtained by direct culture and solid state fermentation on said particles. This is a noteworthy advantage of the invention ; in fact it is not necessary to grind the product as it leaves the fermenter, nor to include any additives.

According to another embodiment of the invention the microorganism is a mushroom entirely in the form of mycelium at the time of spreading, in which case said product is deemed immature, or the mushroom is in the form of mycelium and spores, in which case said product is deemed mature. This control of the mushroom population state, and thus of the efficacy of the product, is an essential characteristic of the invention. To the knowledge of the applicant, the former method does not describe products whose spore and mycelium composition is controlled precisely. The product according to the invention may also comprise a mixture of the mature and immature forms.

According to a further embodiment of the invention said support has a density preferably between 0.6 and 1.

According to another embodiment of the invention the support is of mineral or organic origin, notably montmorillonite, attapulgite or sepiolite. The support must be compatible with a terrestrial or aerial mechanized

application.

According to yet another embodiment of the invention said support has a minimum water retention capacity of 40%.

5

According to another embodiment of the invention said particles have a size of between 100 and 1000 microns and preferably of around 500 microns.

10

Another object of the present invention is a product in which said mushroom is a *Beauveria bassiana* stock, especially the stock registered with the National Collection of Microorganism Cultures under N° I 867, or a mutant of this stock. I-867 was deposited at Institut National de la Recherche Agronomique (I.N.R.A.) on June 7th, 1989.

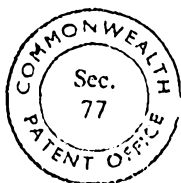
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According to another particular embodiment of the invention said product is dried for storing and spreading.

20

Furthermore, an object of the invention is a process for obtaining an agronomic treatment product with a base of at least one microorganism, to combat the depredation of insect pests and plant diseases, or for fertilization of soils, wherein it comprises direct culture by solid state fermentation of said microorganism on at least one solid support in particles, insoluble in the fermentation medium, and the recuperation of said particles, which are

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colonized by the microorganism, while maturity of the culture is controlled to allow the microorganism to sporulate under the spreading conditions.

5 The association therefore of the microorganism and the solid support particles occurs without intervention of any means or physical and/or chemical agent, which proves especially advantageous for the process according to the invention.

10 Moreover, the microorganisms precolonize the particles during fermentation, ensuring a more effective association of microorganisms and particles.

15 According to an embodiment of the process of the invention the microorganism is a mushroom. Fermentation is stopped when the mycelium is obtained, resulting in a so-called immature product, or when the mycelium and the spores are obtained, resulting in a so-called mature product.

20 Stopping fermentation with a view to producing an immature product allows curtailing of fermentation by several days.

25 According to another embodiment of the process of

the invention said particles have or have not been previously sterilized. This is also an advantage of the invention, since processes according to previous methods require sterilization of supports, to the knowledge of the applicant.

5 This improvement in the fermentation process is thus very significant from the production and economic point of view, because it means that an energy-costly step can be avoided.

10 According to another embodiment of the process of the invention the mushroom used is an entomopathogenic mushroom, effective against one or more crop insect pests. This mushroom can be, in particular, *Beauveria bassiana*, *Beauveria tenella*, *Beauveria brognartii* or *Paecilomyces fumosoroseus*.

15

According to another embodiment of the process of the invention said mushroom is *Beauveria bassiana*. when fermentation take place over 20 to 60 hours, and preferably over 48 hours, it provides an immature product ;
20 when the fermentation time is 90 hours minimum a mature product is then obtained.

25

It will be noted also that the process of the invention can be implemented with increased thickness of support during fermentation, reaching tens of cms in thickness, which is favourable for industrial production as well as



for control of the maturity of the crop.

A further object of the invention is a biological control process against diseases and insect pests in plants, wherein an effective quantity of the above described product, notably with a base of entomopathogenic mushrooms, is spread on plants to be treated, the disease in question being, for example, maize mite which is due to *Ostrinia nubilalis*, but also other mites or other plant diseases.

Yet a further object of the invention is a soil fertilization process, wherein an effective quantity of the fertilizer product with a microorganism base as previously described, is used for spreading.

The following description further illustrates examples of the invention's applications in a non-limiting way, with reference to the attached diagrams in which :

Figure 1 schematically represents a reactor-fermenter which can be used to obtain the product of the invention.

Figure 2 is a graphic representation of the evolution of biochemical parameters as a function of the growth stage following the process described in Example 1.

Figure 3 graphically illustrates results of the assays related to Example 4.

EXAMPLE 1

Production of products according to the invention

a. - Preparation of the inoculum

A semi-synthetic liquid medium A (Table 1) is sown by means of conidia of *Beauveria bassiana*, resulting from a culture on a semi-synthetic medium B (Table 2) aged 5 days, at a rate of 10^6 conidia/ml. This medium is incubated at 25°C in flasks on an agitation table or in a liquid fermenter as a function of quantities of culture to be inoculated.

After 72 hours of growth the medium A contains between 1 and $2 \cdot 10^8$ blastospores/ml.

TABLE 1

Composition of medium A

Cereal maceration liquid	20.0g
Sucrose	30.0g
KNO ₃	5.0g
KH ₂ PO ₄	5.8g
CaCO ₃	2.0g
MgSO ₄ 7H ₂ O	0.1g
Permutated water	1 l

TABLE 2

Composition of medium B

	Glucose	10.0g
	Yeast extract	5.0g
5	KH_2PO_4	0.4g
	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	1.4g
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.6g
	KCl	1.0g
	NH_4NO_3	0.7g
10	Chloramphenicol	0.5g
	Agar	15.0g
	Permutated water	1 1

b. - Preparation of solid state culture medium

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The culture medium used comprises two phases :

- a solid phase constituting the granulose support ;
- a liquid phase constituting the nutritive substratum of the microorganism imbuing the solid phase.

20

The support may be previously sterilized (3 hours at 120°C) or not sterilized ; a sterile process is not essential, since very few contaminants develop fully in the growth conditions utilized and since satisfying results are obtained.

25

This support is impregnated with one of the nutritive media C, D or E (Tables 3, 4 or 5) ; these sterilized nutritive media C, D or E (30 minutes at 115°C) are added from the inoculum at the rate of $3 \cdot 10^6$ blastospores/ml of medium C, D or E.

Impregnation and mixing can be performed in a kneading trough type of apparatus or in the reactor itself, so as to obtain a dry matter rate of around 60% (weight of humid support/weight). Under these conditions an inoculation rate of around $2 \cdot 10^6$ blastospores/g of dry matter is obtained. Where necessary the stock is incubated between 18 and 27°C.

TABLE 3

Composition of medium C

Sucrose	132.7g
Residual non-sugar*	27.0g
Permutated water	1 1

* The residual non-sugar is a by-product from extraction of the sugar composed mainly from nitrogenous substances and commercialized by General Sugar Refineries.

TABLE 4

Composition of medium D

Solulys**	150g
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(Société Roquette)

Permutated water 1 1

** Solulys is a by-product from extraction and refining of corn starch which contains amino acids and lactic acid.

5

TABLE 5

Composition of medium E

Sucrose	124.0g
Solulys	38.5g
Permutated water	1 1

10

The entire homogeneous mixture is transferred to the reactor (Figure 1), if the mixing is not performed there directly.

15

Figure 1 represents the reactor with its monitoring and control device.

20

The reactor comprises a container (2) filled with a semi-solid medium, in which or more return propellers (1) macerate, carried on a mobile trolley (4) and driven by a motor (5).

25

The trolley (4) is equipped with a feed motor (3) and also carries a pulverization ramp (6) fed by solution inlet pipes (19, 20, 21).

Near the bottom of the reactor and parallel to it is an upper perforated plaque (22) constituting a coarse screen which effectively holds the culture medium layer in place ; this layer may be from 0.50 to 1 metre in thickness. There is also a lower perforated plaque (23) forming a finer screen and allowing even distribution of air through the interior of the container (2). The two screens may be replaced to advantage by a single screen comprising a mesh of plaited stainless steel (DYNAPORE plate), serving simultaneously to diffuse air and support the culture medium.

The air enters the fermentation device in the direction indicated by the arrow F2 and exits from it in the direction of the arrow indicated by F1. The air is filtered initially by the filter (18), then, if necessary, cooled with the assistance of the heater battery (17) and humidified in the humidifier (13).

Circulation of air is ensured by the ventilator (15) ; the yield is in the order of $7.51 \text{ mn}^{-1} \text{-kg}$ of dry matter⁻¹.

The mechanism measuring growth conditions in the reactor comprises an oxygen probe (7), a pH probe (8) and a temperature probe (9). Measuring of air temperature and humidity is ensured by probes (11) as air enters and leaves the reactor at (10). Compression strain gauges (12) allow

measuring of growth mass in the reactor, while measuring of air output is performed with the assistance of an output meter.

5 Fermentation control and monitoring of the reactor are ensured by the micro-calculator (24) which receives information supplied by the oxygen probe (7), pH probe (8), temperature probe (9), temperature and humidity probes (10) and (11), compression strain sensors (12),
 10 output meter (14) and which as a function of this information operates the trolley feed motor (3), humidifier (13), ventilator (15), cooler battery (16), heater battery (17) and valves controlling output of the solution inlet pipes (19, 20, 21).

15

During growth the temperature is maintained at around 25°C and growth humidity at around 40% by ventilation of warm, humid air.

20

d. - Control of growth state during production

During growth it is important to be able to follow the evolution of the production, that is, the stage of the mushroom's growth, in order to decide whether to stop growth or not.

25

Following the growth stage it is necessary to be

able to estimate both the mycelium growth and conidiogenesis. The stage following conidiogenesis is carried out meticulously by means of a standardized method for numbering conidia.

5

Method for numbering conidia

Around 1g of culture is weighed precisely and introduced into a flask containing 50 ml of Tween 80 at 1% and about 30g of glass balls of 4mm diameter. The flasks are agitated in a rotary mixer for 20 minutes.

10

The solution thus obtained is diluted by ten in a methylene blue solution. After an hour of incubation at ambient temperature the counts are realized on a Malassez cell.

15

Composition of the methylene blue solution :

Methylene blue	0.6g
KH_2PO_4	81.3g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	5.7g
Water	1.01

20

Estimation of mycelium growth

Direct estimation of mycelium growth cannot be carried out online. However, evolution of respiration can be followed automatically, giving a perfect picture

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of the physiological state of the microorganism.

Measuring the fungal respiration is the only method which at the present time allows the cultures to be followed in real time. Gases are pumped at the outlet of the fermenter, pumped off by means of a 'hygrostop' (Hartman and Braun). Their composition is determined by a URAS 3G type infra-red gas analyser (Hartman and Braun) for the CO₂ and by a Magnos 3 type paramagnetic oxygen analyser (Hartman and Braun). The unit is managed by a microcomputer which calculates different parameters online : the CPR or CO₂ production rate, and the OUR or oxygen consumption rate.

These two parameters are calculated and expressed in mole of CO₂ or O₂ per hour and per gram of dry matter.

Furthermore, pre-performed analyses mean that evolutions of glucosamine and ergosterol rates can be traced.

An example of the result obtained at the time of growth on medium C is shown in Figure 2 in which the growth time has been set out in abscissa of inoculation of the fermenter ; the CPR and rate of glucosamine and ergosterol are set out in ordinates. Curve A represents evolution of the glucosamine rate, curve B evolution of the ergosterol rate and curve C evolution of the CPR.

e. Quantity control of the product obtained at the end of fermentation.

The micrograin simultaneously carries mycelium and conidia. It is difficult to separate the two forms from each other quantitatively, and also to separate the mycelium from the support. Only indirect measuring methods can be performed. These consist of titrating biochemical constituents of mycelium and spores, chitin and ergosterol, after extraction.

Chitin and ergosterol.

Although they allow no online control of the product's maturing, since they are too protracted, these methods are quite satisfactory in characterizing the product at the end of fermentation. Numbering of conidia is carried out in parallel according to the numbering method described above in (d). This method is applicable only from 72 hours of growth, as before this time the spore count is too weak for numbering. Total biomass is estimated by titration of chitin and ergosterol.

Titration of chitin.

Chitin, which constitutes the cell wall, is hydrolyzed in an acid medium and at heat in N acetyl glucosamine (Sakurai et al., 1977, Agric. Biol. Chem. 41, 4, 619-624). Glucosamine reacts after desamination with a coloured reagent, namely methyl 2-benzothiazone hydrazone

(MBTH). Intensity of the blue colouring is proportional to the quantity of glucosamine (Tsuji et al., 1969, Chem. Pharm. Bulletin, 17, 7, 1505-1510 and Ride and Drysdale, 1972, Physiological Plant Pathology, 2, 7, 15).

5

Hydrolysis is performed as follows :

The test sample for processing is first washed three times with a solution of KCl to 1 mole.l⁻¹ to eliminate any interference due to residual nutritive medium ; then 1g of the washed test sample is weighed precisely and 10 ml of concentrated HCl are added. This is left at ambient temperature for 16 hours, then 10 ml of permutated water are added and this solution is sterilized for 2 hours at 130°C.

15

Titration of chitin is performed in two steps :

- Neutralization of the hydrolysate

1 ml of hydrolysate is neutralized by adding 2.5 ml NaOH to 1 mole.l⁻¹ and 1.5 ml permutated water.

20

- Titration

1 ml KHSO₄ at 5% and 1 ml NaNO₂ at 5% are added to 1 ml of the above solution. For 15 minutes the tubes are shaken, then 1 ml NH₄ SO₃ NH₂ at 12.5% is added, after which the tubes are shaken for another 5 minutes. 1 ml

25

MBTH at 0.5% is added and the mixture is put in a bain-marie

for 5 minutes, after which it is cooled ; 1 ml FeCl_3 at 0.5% is added and after 30 minutes the result is calculated at 650 nm.

5 The quantity of glucosamine in the test samples is determined in relation to a range of N acetyl glucosamine (0 to 30 $\mu\text{g/ml}$) realized simultaneously.

10 The results are expressed in μg of glucosamine/g of test sample dry matter.

Titration of ergosterol

Ergosterol, a consitiuent of the cellular membrane, is extracted from the raw product :

15 - by saponification of the product, then by liquid/liquid separation, the sterols are extracted and separated from the fatty acids and all unsaponifiable substances.

20 - by elution in high performance liquid chromatography, the ergosterol is separated from other sterols.

25 - by absorption spectrophotometry in UV, the quantity of ergosterol is determined precisely. Effectively, ergosterol has a specific absorption wavelength of 282 nm and the peak surface obtained on the chromatogram is proportional to the quantity of ergosterol present.

(Seitz et al., 1979, Phytopathology, 69, 11, 1202-1203 ;
 seitz et al., 1977, Cereal Chemistry, 54, 6, 1207-1217 ;
 Kaminski et al., 1983, Fat Science Proceedings, 16th, 1st
 Congress, Budapest).

5

Extraction is performed preactically, as follows :

A quantity of test sample is weighed precisely in a
 globular flask (a sufficient quantity fo the extract to
 contain 0.1 to 30 µg/10 ml). 100 ml methanol and 50 ml
 alcohol potash (KOH 20g ethyl alcohol 50 ml) are added.
 The whole is saponified under reflux for 30 minutes, then
 centrifured for 10 minutes at 6000 t.min.⁻¹. The flotsam
 is mixed with 100 ml water and 100 ml petroleum ether.
 The whole is left in a decanting phial, then the ether
 phase (upper phase) is recuperated and evaporated. The
 dry residue is recovered by 10 ml dichloromethane.

15

The ergosterol is titrated in the extract thus ob-
 tained by high performance liquid chromatography on a
 column lined with Lichrososorb ST 60 silica having a
 grain size of 5µm (Merck). The mobile phase is a mixture
 of methylene chloride - isopropanol (99.5/0.5 V/V). The
 results are expressed in µg of ergosterol per gram of
 dry matter.

20

25

f. - Control of product viability after fermentation.

The titration methods described in (e) allow quantification of the total biomass at the end of the process, but not verification of the viability of the product, either after growth or following a given storage period. Usually, realization of a germination test in Petri dishes can determine viability of a sporulated product, the expressed result being the number of germinated conidia giving birth to a colony in relation to the total number of conidia measured on the Malassez cell.

Given that the microgranulose product contains essentially mycelium, and that there is thus no possibility of any correlation between the mycelian biomass and the number of colonies which have appeared, a method of analysis allowing estimation of the product's viability has been clarified. It concerns the measuring of the aptitude of the mycelium to be sporulated.

The dried growth test sample is humidified and incubated. During incubation the mycelium continues to grow, although this was stopped by drying, and sporulates. After 144 hours of incubation in total, which takes place during growth and the viability test, the conidia count has reached maximum and remains constant. For growth always carried out under the same conditions, the maximum count of conidia is always the same. The conidia count need be

done only after 96 hours of incubation ; the resulting value is an indication of the quality of the product.

Measuring method

5 50g of dried culture are placed in a 500 ml Erlen-
meyer flask and humidified with 22 ml sterile water to
reach a count of dry matter between 60 and 70%. The flasks
are then incubated in a climatized room at 25°C for 96 hours.

10 After this incubation period the conidia count is
determined as described in (d).

g. - Storage

15 The granulose compositions according to the invention
are stored after drying in an atmosphere whose humidity is
controlled by inert gas, such as azote and carbon dioxide,
or under a vacuum preferably at a temperature between 5 and
30°C.

20 EXAMPLE 2

Field comparison of efficacy of microgranules obtained at
various stages of maturity.

25 Preparations of montmorillonite granules of varying
maturity corresponding to incubation times of 48, 72, 96
and 120 hours have been prepared according to the general
method of operation described with reference to Example 1

and have been compared in Table 6.

Efficacy was estimated in a complete block assay with four repetitions. Each plot is infested weekly for three weeks. Each time an ooplaque of mites is introduced per plant.

Efficacy is expressed in percentage of decrease in relation to the proof whose total number is 1.9 caterpillar per plant.

The granules obtained after fermentation of 48, 72 and 96 hours are considered immature, while those obtained over 120 hours are considered mature.

The mixture is composed of 70% granules of 48 hours and 30% mature granules.

It eventuates that immature microgranules, dried before conidiogenesis, are stringly infectious on the ground without the slightest retarding effect.

It should be noted that these immature microgranules contain no infectious propagules (conidia), but are capable of generating them after application in the field.

TABLE 6

Study of granules with controlled maturity

5	Maturity in hours of incubation	Application dose kg/ha	EFFICACY	
			% of decrease number of attacks	% of decrease number of larvae
	48	25	87.3	90
	72	25	75.9	90.8
	96	25	91.7	94
10	120	25	68.2	79.2
	mixture	25	88.1	93.3

EXAMPLE 3

15 Field comparison of efficacy of product according to the
invention and products resulting from other technologies
(liquid or solid formulations).

20 A preparation constituted by a mixture of granules
as defined in Example 2 (70% granules of 48 hours and 30%
mature granules of 120 hours) and obtained in accordance
with the method of operation in Example 1 has been tested
comparatively with formulations prepared according to pro-
cesses described in previous specifications.

25

The assays were carried out on maize fields.

The liquid formulations tested are the following wettable powders :

- a suspension of conidia coated in clay in accordance with the process described in FR-A-2 394 606 ;
- 5 - a suspension of conidia coated in alginate granules, whether added or not, of nutritive elements ;
- a mycelian suspension obtained according to the process described in US-4.530.834.

10 These liquid suspensions are applied to the dose of 10^{13} conidia or 100 g of mycelium, in 200 l, per hectare.

The results of the assays are set out in Table 7.

15 Efficacy was estimated in a complete block assay with four week repetitions. Each plot is infested weekly for 3 weeks. Each time an ooplague of mites is introduced per plant.

20 Efficacy is expressed in percentage of decrease in relation to the proof whose total number is 1.9 caterpillar per plant.

25 These results show that the granulose formulation obtained according to the invention is clearly more effective than the wettable powders tested, with respect to decrease in the number of attacks on plants and decrease in

the larva population.

5 The solid formulations tested comprise two types :
fixation or adsorption of infectious propagules (conidia
or blastospores) on microgranules, whether impregnated or
not, of a nutritive medium. Such a process is specified in
GB-1.002.977 for chemical products. Experimentation results
are set out in Table 8. The conidia were absorbed in sus-
pension in a complete medium with a glucose base (1.5%)
10 and a yeast extract (1.5%).

Efficacy was calculated as previously.

15 These results show a very clear increase in effect-
ively reducing attacks on maize by *Ostrinia nubilalis* and
in reducing the larva population of *Ostrinia nubilalis* at
the time of treatment by the precolonized product according
to the invention.

TABLE 7

Comparative study of granulose formulation with various wettable powders

Formulation	Application dose/ha	Production	Efficacy	
			% decrease in no. of attacks	% decrease in larva population
Granule (mixture)	25 kg	according to invention (montmorillonite)	88.1	93.3
Wettable powder				
. conidia	10^{13}	FR-A 2394.606	3	8.1
. blastospores	10^{13}		0	0
. mycelium	100 g	US 4.530.834	9.3	14
. conidia + 2% chitin	10^{13}	alginate coating	0	28.3
. conidia + 1% glucose	10^{13}	alginate coating	0	8.3
. conidia	10^{13}	alginate coating	0	0
. conidia + 2% chitin + 1% yeast extract	10^{13}	alginate coating	0	15.8

TABLE 8

Effect of precolonization

Preparation	Support	Dose sp/ha	Production	Efficacy	
				% decrease in no. of attacks	% decreases in larva population
ADSORPTION blastospores		10^{13} 10^{12}	US 4.263.036	48 36.3	63 52
ADSORPTION conidia	Expanded pearlite	10^{13}	1 application	36.2	54
		10^{13}	2 applications	52.3	81
		10^{12}	1 application	27	72
Fixing	Montmorill- onite	10^{13} 10^{12}	collage with polyvinyl alcohol	34.1	60.8
PRECOLONIZED GRANULE (mixture)	Montmorill- onite	25 kg/ha	according to the invention	88.1	93.3

EXAMPLE 4Comparison of product according to the invention and chemical reference formulations.

A preparation constituted by a mixture of granules, as defined in Example 2 (70% of granules at 48 hours and 30% of granules at 120 hours), and obtained according to the method of operation in Example 1 was tested comparatively with reference insecticides (Table9).

The chemical reference insecticides are the granule DURSBAN G, which is a chlorpyriphos (made by quinoline), applied at the rate of 25 kg/ha, and DECIS G (Société Pro-cida), applied at the rate of 25 kg/ha.

TABLE 9Comparative efficacy of the product of the invention and chemical reference insecticides.

Product	Application rate	EFFICACY	
		% of decrease in attacks	% of decrease in larva population
GRANULE (mixture on montmorillonite) according to the invention	25	88.1	93.3
DECIS G	25	90	80
DURSBAN G	25	91.3	90.8

Effectiveness in decreasing attacks and larva population were calculated as in the preceding example. These results show no evidence of significant differences between the actions of the product as defined by the invention and the two tested insecticides ; this shows that the product as defined by the invention has an effect at least comparable to those of these two products.

Tests were made on remanences in the field of the insecticide effect of the mature and immature products of the invention and on the chemical formulation DECIS G.

In Figure 3 the time after the start of the experiment has been set out in abscissa, expressed in days, and decrease in the larva population has been set out in ordinates, expressed in percentage.

The curves A, B and C represent respectively evolution of the insecticide effect of the immature and mature granules as defined by the invention is comparable to that of the chemical insecticide DECIS G, which is a reference product.

EXAMPLE 5

Comparison of efficacy of granulose supports.

Preparations obtained by the general method of

operation described in Example 1 by fermentation as far as
the mature stage of *Beauveria bassiana* on various supports
were tested for their efficacy in decreasing attacks on
plants and decreasing the larva population. The results are
5 summarized in Table 10 ; their effectiveness in decrease
was calculated as in the preceding examples.

These results show that expanded perlite and ver-
miculite permit good production of germs with respect to
10 effectiveness in decreasing attacks on plants and the number
of larvae.

However, these two supports are incompatible with
the mechanized application. Chamotte and artal are less
15 efficacious. Montmorillonite is the support which provided
the best results for production and effectiveness in
application.

The invention has been illustrated in the above in
20 its particular application to the mushroom *Beauveria*
bassiana. It is understood, however, that it is in no
way limited to this particular type of mushroom.

TABLE 10

Comparison of efficacy of granulose supports

SUPPORT	APPLICATION DOSE kg/ha	CHARACTERISTICS		EFFICACY	
		size	density in mn	% decrease in no. of attacks	% decrease in larva population
Expanded pearlite and vermiculite	25	1-2	0.1	86.8	90
	2.5	-	-	79.6	86.7
Montmorillo- nite (mature granules)	25	0.5-1	0.6	68.2	79.2
Chamotte (mature)	25	0.5-1	1.35	64.2	70.0
Artal (mature)	25	0.5-1	1.2	38.4	70.0

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A plant protection composition containing particles formed from at least one solid support carrying at least one microorganism effective against insect pests or crop diseases or for soil fertilization, said particles being colonized by the microorganism, the latter having an aptitude to sporulate under spreading conditions.
2. A composition as claimed in Claim 1, wherein it can be spread in a field immediately after it is obtained by direct growth and solid state fermentation on said particles.
3. A composition as claimed in either of Claims 1 and 2, wherein the microorganism is a mushroom.
4. A composition as claimed in Claim 3, wherein the mushroom is entirely in mycelium form at the time of distribution, in which case said product is considered immature.
5. A composition as claimed in Claim 3, wherein the mushroom is in the form of mycelium and spores, in which case said product is considered mature.
6. A composition as claimed in Claim 3, wherein it comprises a mixture of immature and mature forms.
7. A composition as claimed in any of Claims 1 to 6, wherein said support has a density between 0.6 and 1.
8. A composition as claimed in any of Claims 1 to 7, wherein said support is of mineral or organic origin.
9. A composition as claimed in Claim 8, wherein said support is selected from montmorillonite attapulgite.
10. Product as claimed in any of Claims 1 to 9, wherein said support has minimum water retention capacity of 40%.
11. Product as claimed in any of Claims 1 to 11, wherein said particles are of a size between 100 and 1000 microns, preferably around 500 microns.
12. Stock of *Beauveria bassiana* registered with the National Collection of Microorganism Cultures under No. I 867.
13. Stock of *Beauveria bassiana*, wherein it results from one or more mutations of the stock as claimed in Claim 12. *and has the same activity as*
14. Product as claimed in any of Claims 3 to 11, wherein the mushroom used is an entomopathogenic mushroom, effective against one or more crop insect pests.
15. Product as claimed in any of Claims 3 to 11 and 14, wherein the mushroom is *Beauveria bassiana*.



16. Process for preparing a product to combat insect pests or plant diseases or for soil fertilization, which comprises direct growth by solid state fermentation of at least one microorganism on at least one solid support in particles, insoluble in the fermentation medium and the recuperation of said particles which are colonized by the microorganism, maturing of the growth being controlled to allow the microorganism to sporulate under distribution conditions.

17. Process as claimed in Claim 16, wherein the microorganism is a mushroom.

18. Process as claimed in Claim 17, wherein fermentation is stopped when the mycelium is obtained, resulting in a so-called immature product.

19. Process as claimed in Claim 17, wherein fermentation is continued until the mycelium and spores are obtained, resulting in a so-called mature product.

20. Process as claimed in any of Claims 17 to 19, wherein the mushroom used is an entomopathogenic mushroom, effective against crop insect pests.

21. Process as claimed in any of Claims 17 to 20, wherein the mushroom is *Beauveria bassiana* and fermentation takes place over 20 to 60 hours, preferably 48 hours, in which case an immature product is obtained.

22. Process as claimed in any of Claims 17, 19 and 20, wherein the mushroom is *Beauveria bassiana* and fermentation takes place over a minimum of 90 hours, in which case a mature product is obtained.

23. A method of protecting plants against diseases or insect pests, wherein an effective quantity of the product as claimed in any of Claims 1 to 11 and 14 is spread on plants requiring treatment.

24. Process as claimed in Claim 23, wherein plants are treated against *Ostrinia nubilalis*, known as maize mite.

25. Soil fertilization process, wherein an effective quantity of the product as claimed in any of Claims 1 to 11 is spread on soils requiring treatment.

DATED this 27th day of June 1990.

INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE (I.N.R.A.)

WATERMARK PATENT & TRADEMARK ATTORNEYS
"THE ATRIUM"
290 BURWOOD ROAD
HAWTHORN. VIC. 3122.

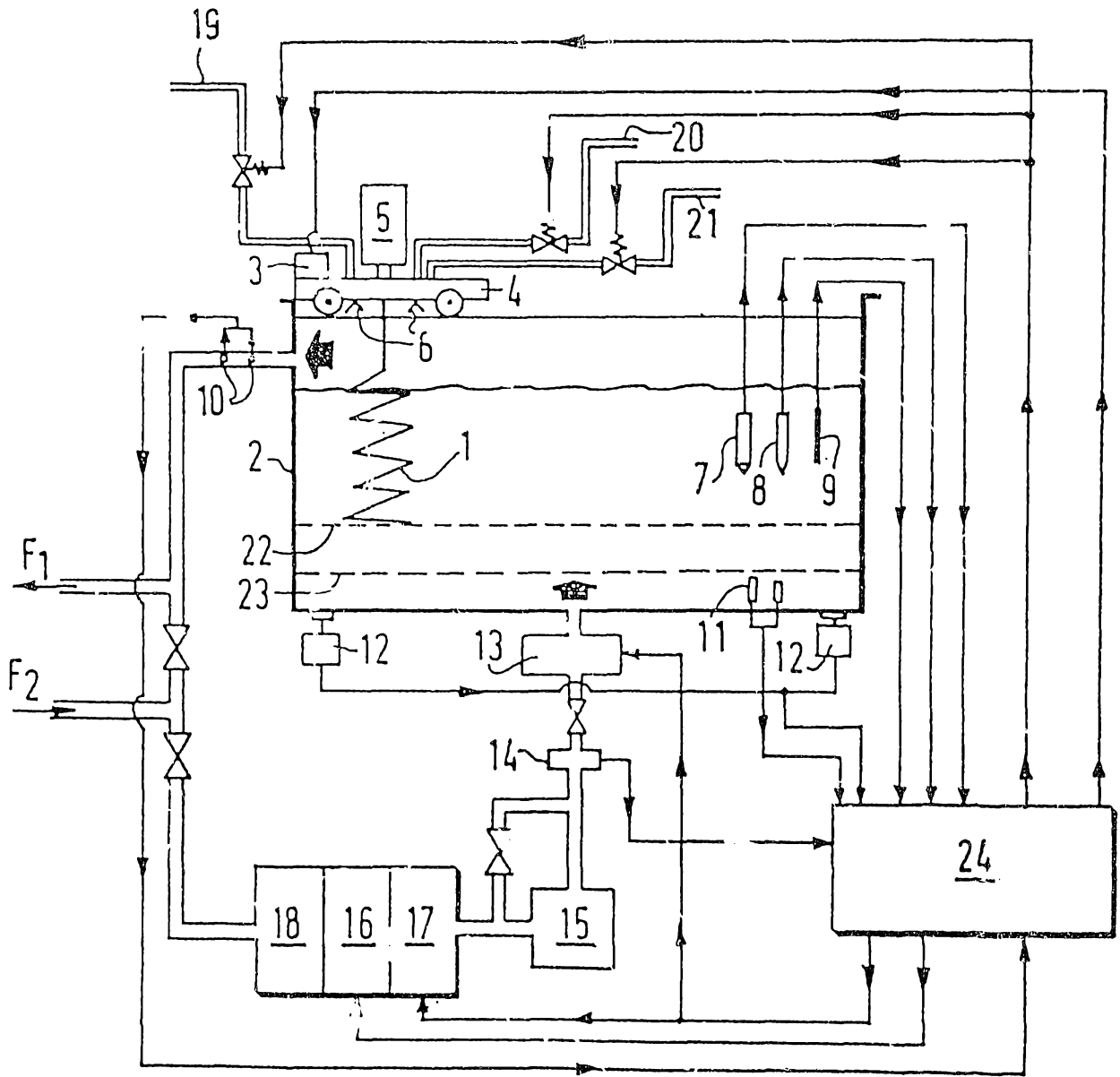


FIG 1

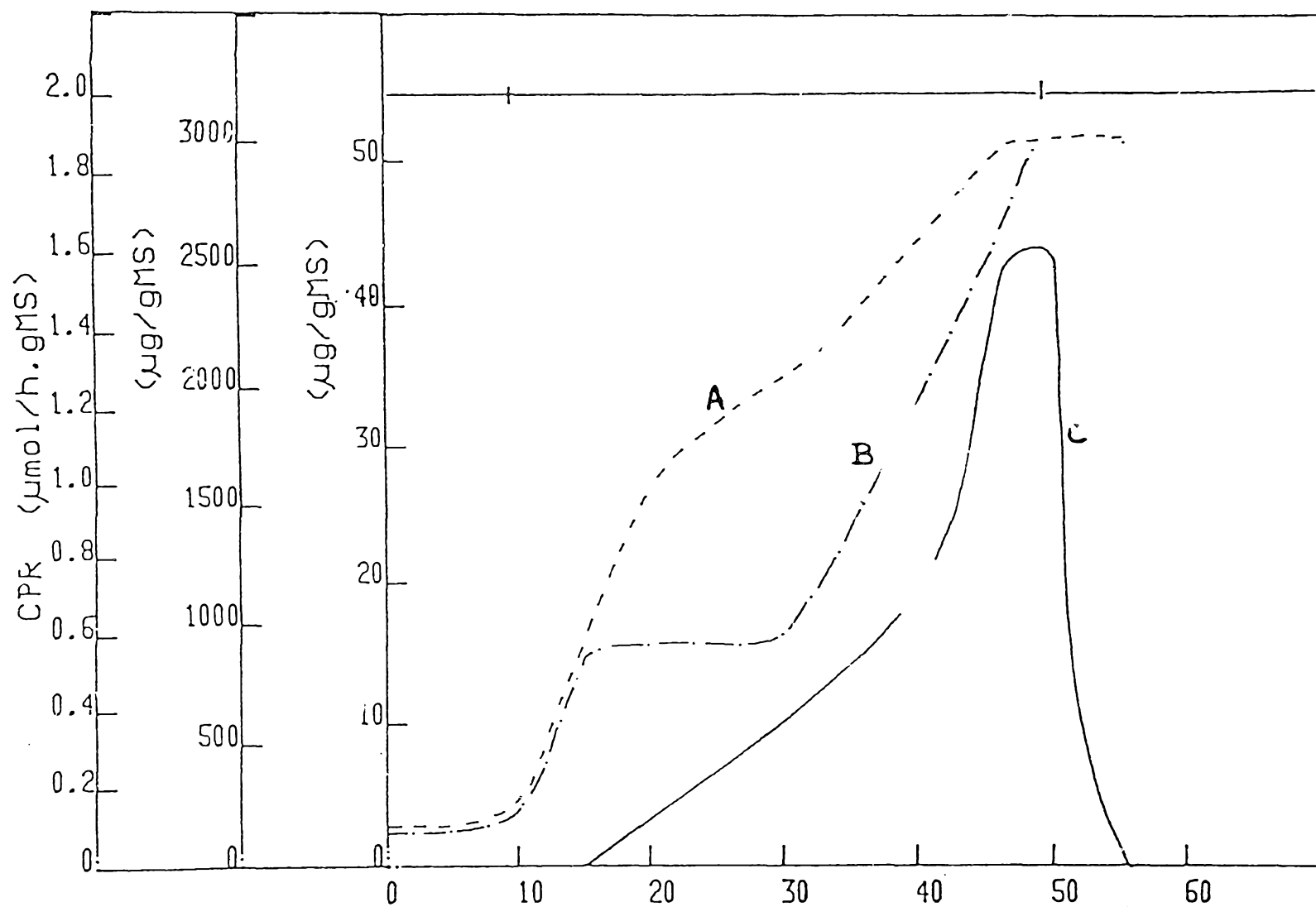


FIG 2

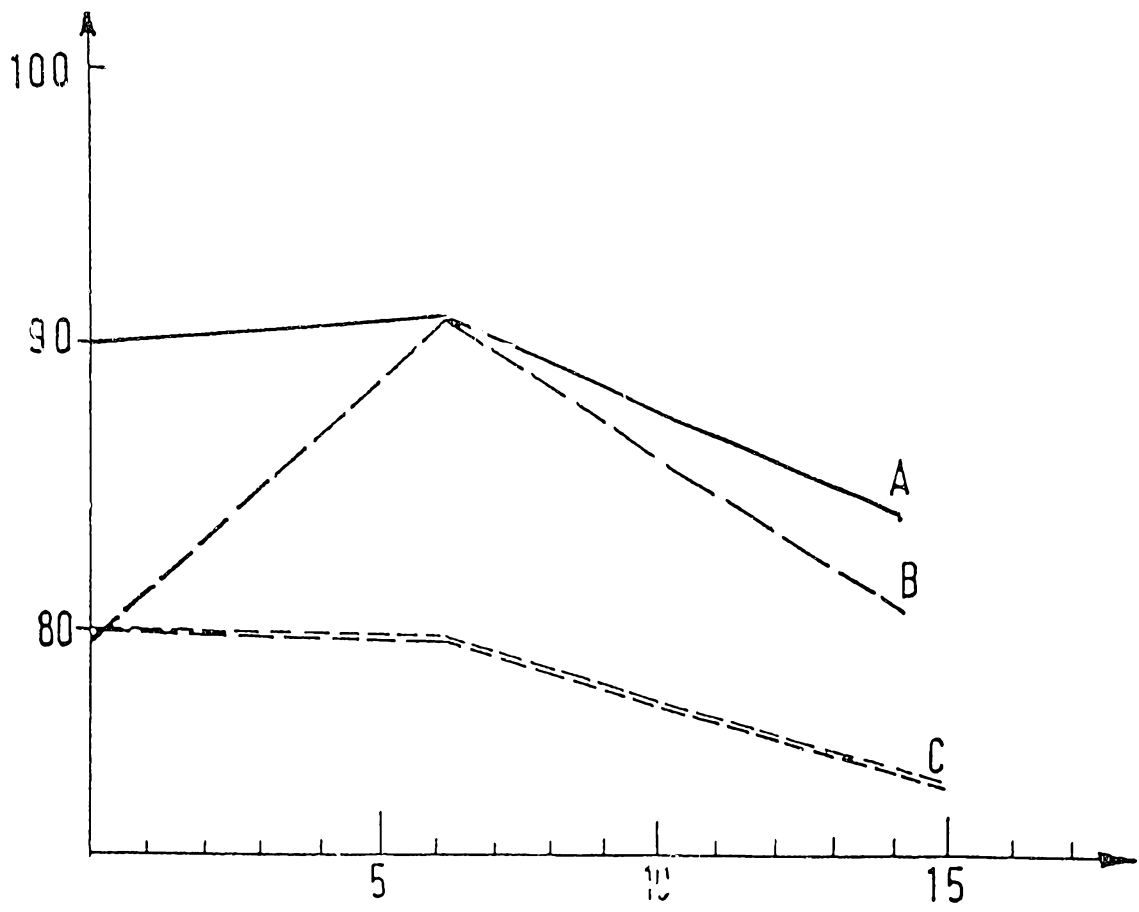


FIG 3