

MICROCIDAL COMPOSITION FOR THE CONTROL OF CONTAMINANTS IN PRODUCTION PROCESSES OF IN VITRO PLANTS

DESCRIPTION

5 This invention is related to a branch of Agricultural Biotechnology and consists in the application of a formulation with active principle 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene as chemical sterilizer to solid culture media for the control of bacterial and fungal contaminants in production processes of in vitro plants.

These contaminations cause substantial losses to the whole process since the autoclave only eliminates at-the-moment pollutions. In some cases different antibiotics, e.g., Cefotaxim, Novobiocin, and Dihydrostreptomycin have been used to prevent contamination without satisfactory results.

The Cuban Certificate of Author of Invention No. 22446 describes the antibacterial and antifungal activities of 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene against strains isolated from hospitals and veterinary institutions.

20 The main limitation against the use of this active principle was its close-to-null water solubility and the also reduced dissolution rate in an aqueous medium. A possible solution to this problem could be solid dispersions, which allow for a decrease of the particle size to molecular level.

25 The system proposed consists in dispersion by fusion or coprecipitation of the active principle in a kit physiologically inert and easily soluble in water, which is formed by high molecular weight polyethylene glycols and other polymers.

Dispersion by fusion:

30 The selection of the manufacture methods and temperature parameters, as well as that of stirring times is based on the melting point and thermal stability of the active principle and the polymer elected.

1-(5-bromofur-2-yl)-2-bromo-2-nitroethene lets without decomposition at a temperature of 89-90 °C and is thermally stable up to 110 °C.

The physical mixture of the active principle and PEG-6000 in a ration from 1:20 to 1:1 heats up to fusion at the temperature of 60-80 °C and remains under constant stirring for 15 minutes until

The chemical stability of the active principle dissolved-dispersed in polyethylene glycol has been studied with the use of Gas Chromatography as the analytical technique for the quantification thereof. A chemical stability study lasting 385 days proves the no existence of decomposition.

Table No. 2: Evaluation of the shelf chemical stability of solid dispersion in PEG-6000 at 30% by Gas Chromatography

Time (days)	0	60	90	120	150	210	306	385
% a.p.	28.6	28.9	29.9	28.8	29.0	29.0	29.3	28.9

Following the completion of formulation studies an in vitro assay was conducted to determine the product activity against agricultural pathogens and contaminating microorganisms present in biotechnology production processes. The study of bacteria was carried out under the serial dilution method in tubes (Bailey and Scott, 1974 as modified by E. Silveira et al., 1986) and the action against fungi was evaluated through the diameter growth of the colony (Casadens et al., 1985).

The concentrations used for evaluating the bioactive product behavior were 1 to 10 µg/ml. The culture medium, temperature and interval of incubation were adjusted to the conditions and characteristics of the microorganism under assay.

The LD₅₀ was calculated in µg/ml under Probit's method.

After proving the active principle in vitro action against fungal and bacterial contaminants of the in vitro planting of bananas, potatoes, and sugar cane we proceeded as follows:

An effective washout of the flasks and lids to be used in the production of in vitro plants was conducted using detergent, rinsing them with sufficient water and then submerging the bottles in a sodium hypochlorite solution at 0.5% for a time interval never smaller than 3 minutes. Afterwards, the flasks are placed upside down and they should be used totally dry before 24 hours.

Once the culture medium is prepared and reaches a temperature of approximately 60-80 °C it is added the necessary weight of the formulation to obtain a concentration of 35 µg/ml and, then the

an entire homogeneity of the melting is reached. Then, the mixture melted is poured and spread as a thin layer on refrigerated stainless steel plates, thus ensuring the speed of the solidification process. The solid obtained is grinded in
5 mills and screened at a particle size smaller than 0.2 mm.

The melting point range of solid dispersions of the active principle and PEG-6000 varies with the composition of the mixture, being 53-54 °C for the solid dispersion at 30% whose composition coincides with the eutectic composition of the binary
10 system active principle-PEG-6000.

Dispersion by co-precipitation

The elaboration of solid disperse systems of the active principle with polymers whose melting points are higher than 110 °C affect the active principle thermal stability and, therefore, it is
15 recommended the co-precipitation method in a solvent.

The mixture of the active principle and the polymer in ratios from 1:20 to 1:1 is dissolved in a polar solvent heated in an interval of 10 - 60 minutes at a temperature ranging between 40
20 and 60 °C. Then, it is vacuum-rotoevaporated controlling temperature from 40-80 °C. The solid thus obtained is grinded in a mill and screened at a particle size smaller than 0.2 mm.

Important in the obtainment of aqueous homogeneous solutions/suspensions of the active principle are the dissolution
25 properties thereof. Table No. 1 establishes a comparison among the dissolution rates of the active principle with humectant, micronized, and standard particle sizes and using a solid dispersion with PEG-6000 at 30%. It may be noticed how the active principle properties improve significantly thus ensuring the rate
30 and homogeneity of its distribution in the culture medium.

Table No. 1: Dissolution rate evaluation of the active principle in different forms

	Times		
	10 %	50 %	90 %
Active principle particle size: 0.1-0.2 mm	<1 h	<12 h	<48 h
Micronized active principle	<30 min.	<6 h	<24 h
Humectant active principle	<15 min.	<3 h	<6 h
Solid dispersion a.p. -PEG-6000 at 30%	<5 s	<10 s	<15 s

volume required is poured into each of the flasks proceeding to the planting procedure after 24 hours.

This concentration is selected on the basis of its effectivity and lack of phytotoxic effects in the culture media studied.

- 5 The range assayed was 1-50 $\mu\text{g/ml}$. The behavior of in vitro cultures of bananas, potatoes, and sugar cane was evaluated after 72 hours and during the period required for its culture in order to carry out the following sub-culture in the multiplication medium. Such a study was conducted in the rootage culture medium.
- 10 Out of these assays it possible to infer that contaminations behaved similarly to those of the witness used with physical sterilization of the culture medium using an autoclave and that they started increasing as from the eleventh day. In addition, an increment of the multiplication coefficient was noticed when
- 15 working with the chemical sterilizer.

The rootage medium without autoclaves nor addition of hormones stimulating the induction of roots showed a visible effect superior to that of the standard method with the appearance of stronger and more abundant roots in a shorter period of time. The

20 contamination percentage detected was significant.

Phytotoxicity was evaluated considering necrosis, chlorosis, multiplication coefficient, and junction-to-junction distances (for potatoes). The optimum dose in the three cultures studied was 35 $\mu\text{g/ml}$.

- 25 Example 1: Influence of temperature and time on the purity of the solid dispersion obtained by the fusion method.

Temperature (°C)	Time (min.)	Purity (%)
80	15	99.89
80	30	99.61
80	60	99.22
100	15	99.00
100	30	98.35
100	60	97.21
120	15	90.58
120	30	78.19
120	60	63.85

Example No. 2: In vitro tests conducted with relevant LD50 value against the following agriculturally important microorganisms

1.1. Gram Negative Bacteria	LD50 µg/ml
1.1.1. Pseudomonadaceae family	
Xanthomonas vesicatoria	15-25
Pseudomona cichorii	15-25
1.1.2. Enterobacteriaceae family	
Erwinia Chrysanthemi	20-30
Gram Negative Bacillus (bioplants contaminant)	20-30
1.2. Gram Positive Bacteria	
1.2.1. Bacillaceae family	
Gram Negative Bacillus (bioplants contaminant)	20-30
2.1. Monomorphic yeastlike fungi	
2.1.1. Dematiaceae family	
Alternaria porri	1.9-2.5
Alternaria solani	1.5-2.3
2.1.2. Pythiaceae family	
Phytophthora parasitica	1.9-3.2
2.1.3. Tuberculariaceae family	
Fusarium oxysporium	10.4-11.5
2.1.4. Melanconiaceae family	
Colletotrichum gloesporioides	1.0-1.8
2.1.5. Estilbaceae family	
Sclerotium rolfii	0.2-0.9
2.1.6. Dothideaceae family	
Mycosphaerella muscicola	3.0-7.0
Mycosphaerella fijiensis	3.0-7.0
2.1.7. Moniliaceae family	
Aspergillus niger	15.5-17.5
Penicillium sp	15.6-18.5
Cladosporium sp	10.6-11.4

5 Example No. 3: Action of the solid dispersion containing 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene against contaminating organisms in culture media from bioplants

Two scaleup studies were conducted on a Musa sp culture in the multiplication phase with the following results:

Scaleup No.	Clone	Explant No.	Contamination % before day eleven	Contamination % after day eleven
1	Gran nayne	8240	1.72	4.67

2	FHIA-03	15000	3.92	7.26
Witness	FHIA-03	15000	4.00	5.80

The death rate percentage during the three assays was 0.81% for the scaleups conducted adding the active principle and 1% for the standard method.

- 5 Example No. 4: Action of the solid dispersion containing 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene on the development of in vitro plants.

Two scaleup studies were conducted on a Musa sp culture in the multiplication phase with the following results:

10

Scaleup No.	Clone	Explant No.	Multiplication coefficient
1	Gran nayne	8240	3.87
2	FHIA-03	15000	4.03
Witness	FHIA-03	15000	3.0

15

In addition, an assay was conducted on Musa sp. With clones Gran nayne and FHIA-03 in rootage culture medium without adding root-inducing hormones (A.I.A.). Both clones behaved better in the variants where a chemical sterilization was carried out since a larger amount of stronger roots was observed after 5 days, while the witness with the standard procedure only showed a small radical development in the same period.



MICROCIDAL COMPOSITION FOR THE CONTROL OF CONTAMINANTS IN
PRODUCTION PROCESSES OF IN VITRO PLANTS

CLAIMS

- 5
1. Chemical composition formed by a water-soluble polymer and active principle 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene as chemical sterilizer to inhibit microbial growth in the production process of in vitro plants of different crops.
 - 10 2. A chemical composition as in Claim No. 1 where the method to be used is solid dispersion.
 3. A chemical composition as in Claim No. 1 where the method to be used is solvent co-precipitation.
 - 15 4. A chemical composition as in Claim No. 1 where the polymers used are high molecular weight polyethylene glycols (PEG-6000-20000).
 5. A chemical composition as in Claim No. 4 where PEG-6000 is preferably utilized among the high molecular weight polyethylene glycols to be used.
 - 20 6. A chemical composition as in Claim No. 3 where the solvents to be used are polar.
 7. A chemical composition as in Claim No. 6 where some of the solvents used are ethanol and chloroform.
 - 25 8. Use of the chemical composition described in Claim No. 1 for the substitution of hormones in the rootage process of in vitro plants of crops, e.g. Musa sp.

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5 Abstract.

A microcidal composition consisting of a water-soluble polymer and the active principle 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene is proposed to guarantee the control of contaminants
10 in the production processes of in vitro plants without having to utilize the usual autoclave sterilization procedure.

This process shows that the addition of the product to the culture medium without autoclaving it does not require growth
15 hormones for the rootage, with results similar to those obtained by adding hormones and using the autoclave



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CLAIMS

1. Microbicidal composition comprising 1- (5-bromofur-2-yl) -2-bromo-2-nitroethene and also containing a water-soluble polymer.
2. Microcidal composition of claim 1 wherein the polymer used is a high molecular weight polyethylene glycol.
3. Microbicidal composition of claim 2 wherein the high molecular weight polyethylene glycol used is preferably of molecular weight between 6000 and 20000.
4. Microbicidal composition of claim 3 wherein said polyethylene glycol is PEG 6000.
5. Method for obtainment the microbicidal composition of claim 1 wherein it is used a method of solid dispersion.
6. Method according to claim 5 wherein the composition is obtained by dispersion by fusion.
7. Method according to claim 4 wherein the composition is obtained by co-precipitation using solvents.
8. Method according to claim 7 wherein the solvents used are polar solvents.
9. Method according to claim 8 wherein the solvents used are ethanol, chloroform, methanol or isopropyl alcohol, among other solvents.
10. Use of a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a high molecular weight polyethylene glycol as sterilizing agent for the control of contamination of culture media.
11. Use of a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a high molecular weight polyethylene glycol as sterilizing agent for the control of contamination in culture media used in the production of *in vitro* cultivated plants.
12. Use of a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a high molecular weight polyethylene glycol as rootage

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agent in culture media used in the production of "in vitro" cultivated plants.

13. Method for sterilization of culture media used in the production of "in vitro" cultivated plants wherein it is used as sterilizing agent a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a high molecular weight polyethylene glycol.
14. Method according to claim 13 wherein said sterilizing agent is used at the same time as rootage agent in culture media used in the production of "in vitro" cultivated plants.
15. Method according to claims 13 and 14 wherein the optimum dose of 1-(5-Bromofur-2-yl)-2-bromo-2-nitroethene used is in the range of 30 and 45 $\mu\text{g/ml}$.

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THE CLAIMS

1. Microbicidal composition containing 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and also containing a water soluble polymer.
2. Microbicidal composition of claim 1 wherein the water soluble polymer used is a high molecular weight polyethylene glycol.
3. Microbicidal composition of claim 2 wherein the high molecular weight polyethylene glycol used is preferably of molecular weight between 6000 and 20000.
4. Microbicidal composition of claim 3 wherein said polyethylene glycol is PEG 6000.
5. A method for obtainment of the microbicidal composition of claim 1 comprising the step of applying a process of solid dispersion to a quantity of 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene.
6. A method according to claim 5 wherein the process of solid dispersion is dispersion by fusion.
7. Method according to claim 6 comprising the steps of:
 - Preparing a mixture of 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene, an active principle and PEG-6000;
 - Heating the mixture to about 60-80°C;
 - Stirring the mixture until homogeneity of melting is reached;
 - Pouring the melted mixture over a solidification medium;
 - Spreading the melted mixture as a thin layer over the solidification medium; and
 - Obtaining a solid.
8. Method according to claim 5 wherein the process is a process of co-precipitation using solvents.
9. Method according to claim 8 including the steps of:
 - Dissolving a mixture of 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene, an active principle and a polymer in a polar solvent.
 - Vacuumrotoevaporating the mixture; and
 - Obtaining a solid.
10. A method according to claims 8 or 9 wherein the solvents used are polar solvents.
11. A method according to claim 10 wherein the solvents used are selected from the group comprising ethanol, chloroform, methanol or isopropyl alcohol.
12. Use of a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a water soluble polymer as sterilizing agent for the control of contamination of culture media.
13. Use according to claim 12 in association with the production of in-vitro cultivated plants.
14. Use of a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a water soluble polymer as rootage agent in culture media for the production of in-vitro cultivated plants.
15. Use according to any one of claims 12 to 14 wherein the water soluble polymer is a high molecular weight polyethylene glycol.
16. A method for sterilization of culture media used in the production of in-vitro cultivated plants including the step of adding a composition comprising 1-(5-bromofur-2-yl)-2-bromo-2-nitroethene and a water soluble polymer.
17. A method according to claim 16 wherein water soluble polymer is high molecular weight polyethylene glycol
18. A method according to claim 16 wherein said sterilizing agent is too as rootage agent in culture media used in the production of in-vitro cultivated plants.
19. A method according to claims 16 to 18 wherein the optimum dose of 1-(5-Bromofur-2-yl)-2-bromo-2-nitroethene used is in the range of 30 and 45 ug/ml.

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