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Biological Effect of Two Strains of Microorganisms Antagonistic to *Botrytis Cinerea* : Causal Organism of Gray Mold on Strawberry

التأثير البيولوجي لسلالتين من الكائنات الحية الدقيقة المضادة للفطر المسبب لمرض العفن الرمادي في التوت الأرضي / الفراولة

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Abstract

Trichoderma sp. (strain CI306) and *Bacillus amyloliquefaciens* (strain GA1) have shown antagonistic effect against *Botrytis cinerea.* These strains were formulated in an invert emulsion (water - in - oil). Conidia of strain CI306 or endospores of strain GA1 were firstly suspended in water, then introduced into the invert emulsion at the time of mixing the ingredients. Invert emulsions developed in this study and designated IE.1 and IE.3 have the following characteristics: i) a high stability, with an emulsification rate of 99 to 100%; ii) a low and constant viscosity of 25 centipoises for IE.1 and 35 centipoises for IE.3; iii) a high water content ranging from 20.4 to 34.6% for IE.1 and from 16.8 to 18.6% for IE 3 after 24 hours of application at 25°C and 66 to 81% atmospheric relative humidity respectively; and iv) an excellent survival of tested microorganisms after introduction into invert emulsions especially in IE.1. Moreover, the ingredients of invert emulsion IE.1 especially the oil (Radia 7108) and the emulsifier (Radiasurf 7155) are used as food additives in Belgium. Biological efficacy of the two strains against *B.cinerea* was assessed using foliar discs of strawberry. Lesion development due to *Botrytis* was significantly reduced on treated foliar discs with these strains when were applied in formulated form at low levels of relative humidity in comparison with the non-fomulated form. *Trichoderma*-IE.1 formulation, for example, reduced the lesion development at 45% compared to non-formulated form (MDBLCC=0.80 and 0.44 in formulated and non-formulated forms respectively). In addition, the number of *Botrytis* conidiophores appeared on infected surfaces was greatly inhibited on treated foliar discs with strains compared with the non-treated control. *Trichoderma* - IE.1 fomulation, for example, decreased the number of

Botrytis conidiophores to nil compared to non-treated control (MRDBC=0/+++ or 0/++ according to relative humidity level).

Key words: Antagonist, *Trichoderma* sp., *Bacillus amyloliquefaciens*, Gray mold, *Botrytis cinerea*, Invert emulsion, Biological efficay, Mixing technique, Oil phase, Aqueous phase, Strawberry.

تم عمل مستحضرات من كل من السلالة CI306 من الفطر المضاد (*Trichoderma* sp) والسلالة GA1 من البكتيريا المضادة (*Bacillus amyloliquefaciens*) على شكل مستحلبات منعكسة (Invert emulsions) عن طريق إدخال المحاليل المعلقة لهاتين السلالتين الى المستحلبات المنعكسة عند تحضيرها . امتازت المستحلبات المنعكسة المستخدمة والتي تم تحضيرها تبعا لتركيبة جديدة بكونها ذات ثباتية عالية ولزوجة منخفضة وثابتة وبمقدرتها على الاحتفاظ بنسبة عالية من الماء بعد ٢٤ ساعة من استعمالها بالاضافة الى كونها ملائمة ولا تؤثر في حيوية الكائنات الحية الدقيقة التي تم ادخالها لهذه المستحلبات . تمت كذلك دراسة الفاعلية البيولوجية لهاتين السلالتين المضادتين للفطر المسبب لمرض العفن الرمادي (*Botrytis cinerea*) على التوت الارضي/ الفراولة باستخدام اختبار خاص تم وضعه للعدوى بالفطر المسبب للمرض والكائنات المضادة على اقراص من ورق التوت الارضي / الفراولة حيث اثبتت هذه الكائنات المضادة مقدرتها على تثبيط اتساع او امتداد قطر بقعة المرض الناتجة عن العدوى بالفطر المسبب للمرض من جهة وكذلك تقليل عدد الحوامل الكونيدية الخاصة بهذا الفطر في مناطق الاصابة من جهة اخرى مقارنة بالشاهد .

Introduction

Gray mold is one of the destructive plant diseases that has a wide host range under field and postharvest conditions. Its causal organism (*Botrytis cinerea* Pers ex Fr) infects preferably plant organs at ageing stage and fruits after harvesting [1,2]. One of the control measures involved in the disease management is the use of biocontrol agents (bacteria, fungi and yeasts) which showed promising results in the recent years [3-8]. Microbial biocontrol agents of plant pathogens have specific requirements for free water, in form of dew or rain, to achieve good control. Moreover, an extended period of exposure to free water is often required for maximum control. Therefore, there has been considerable interest in developing formulations of these microorganisms that enhance microbial survival in the absence of free moisture. One of these formulations involves the use of

water -in -oil (invert) emulsion. This type of emulsions has shown promise as a carrier for weed-infecting fungi (mycoherbicides) and as a water source to facilitate fungal infection [9-11].

Many examples on mycoherbicides formulated in invert emulsion were cited in literature: an invert emulsion replacing the dew period requirment of *Alternaria cassiae* (Sacc.) Rands conidia was successfully applied to control the sicklepod (*Cassiae obtusifoliae L.*) [12]. For significant germination of the fungus, it was necessary to maintain water content of this invert at 10% over a period of 18 hours [11]. Another invert emulsion with improved physical properties has an oil-phase containing a paraffin wax, a paraffin spray oil and an unsaturated monoglyceride emulsifier [13]. This invert emulsion retarded evaporation, retained a film of water around the conidia of introduced fungi for a sufficient period to allow germination and penetration to take place. Finally, an invert emulsion reduced the inoculum threshold required for infection of *Cassiae obtusifolia* and *Datura stramonium L.* by *Alternaria cassiae* and *A.crassa* (Sacc.) Rands, respectively [9]. Among the different commercial mycoherbicides, few are formulated in an oil adjuvant including casst ® containing *Alternaria cassiae* which is applied to control both sicklepod (*Cassiae obtusifolia*) and coffee sena (*Cassiae occidentalis L.*) in soybean and peanut fields [14].

In spite of the development of other types of formulations for microorganisms antagonistic to phytopathogens, especially for *Trichoderma* spp., we are not aware of any invert emulsion formulation that has been used as a carrier for these biocontrol agents. Therefore, the objectives of this research were: i) to develop stable, non viscous invert emulsions (water-in-oil formulation), then to introduce two strains of microorganisms antagonistic to the causal agent of gray mold (*Botrytis cinerea*) into these emulsions; ii) to test the compatibility of these invert emulsions with introduced microorganisms; iii) to determine the water content of these invert emulsions after 24 hours of application; and iv) to test the biological efficacy of two strains of microorganisms antagonistic to *Botrytis cinerea* on strawberry foliar discs before and after their introduction into the invert emulsions.

Materials and Methods

For conducting experiments, bacterial endospores of *Bacillus amyloliquefaciens* (strain GA1) and conidia of *Trichoderma* sp. (strain CI306) were obtained by subculturing GA1 on Potato Dextrose Agar (PDA) and CI306 on Oat Meal Agar (OMA) successively, then incubating petri-dishes for 4 days in case of CI306 and 2 days in case of GA1 at 25° C with 16 hours of illumination. The number of conidia in the suspension was determined by a haemocytometer. Endospores of the bacteria were obtained by treating the bacterial suspension with hot water (85° C) for 10 minutes, the concentration of endospores (viable and non-viable) in the treated suspension was determined by measuring the optical density at 595 nm wave length, then using the calculated equation of linear relationship between the number of colony-forming-unit (CFU) per ml of bacterial suspension and the optical density (Y=0.17620X+0.13624, where Y=optical density and X = CFU / ml). For the pathogen (*B.cinerea*, strain B20), incubation after subculturing and determination of the concentration of prepared suspension were identical to *Trichoderma*.

Invert emulsions involved in the experiments were selected from several formulations of invert emulsion prepared from 5 types of oils and 6 types of emulsifiers. Selected invert emulsions designated IE.1 and IE.3 were composed of the following ingredients: i) aqueous phase of IE.1: 1% wax[b], 10% glycerin[d] and 89% distilled water; ii) oil phase of IE.1: 5% emulsifier[c] and 95% oil[e]; iii) aqueous phase of IE.3: 1% wax[b], 10% glycerin[d] and 89% distilled water; and iv) oil phase of IE.3: 5% emulsifier[f] and 95% oil[g]. The criteria of selection were: low viscosity and high stability. In these preparations, the quantity of aqueous phase was gradually increased from 10 to 50% of final ingredients which resulted in improving stability and viscosity. Moreover, during the prepration, the aqueous phase was always added into the oil phase at a ratio of 1:1(w/w), then the two phases were mixed together at a constant and high speed (1200 rpm for 1 minute) using a mixer of ultra-turrax type.

The two strains of antagonists were, at first, introduced individually into the aqueous phase (AP) by substituting the quantity of water in this phase (89%) by the suspension of these strains, then the water soluble wax was

added to the aqueous phase (1% by weight) and then heated for two minutes at 50° C using a water-bath before adding glycerine (10% by weight). This phase was then mixed with oil phase using a mixer as described above. The concentration of *Trichoderma* and *Bacillus* strains in the invert emulsions were: 1 x 10^8 conidia/ml and 5 x 10^7 CFU / ml respectively.

The viscosity of the invert emulsions was measured with a viscometer (Brookfield Synchro-electric viscometer: model LVT, serial number 56673). Ten subsamples of each emulsion (100-200ml according to the type of the spindle) were regularly used to measure viscosity. During this measurment, emulsions were stored at 25 ± 2° C for 7 weeks using 100ml screw-capped graduated cylinders. The dial reading appeared on the viscometer scale which resulted from the spindle movement inside the invert emulsion is multiplied by a conversion factor to give the emulsion viscosity.

The stability of invert emulsions was determined by measuring the emulsification rate of the two phases composing the emulsion that could be obviously obeserved in 100ml screw-capped graduated cylinders with the aqueous phase at the bottom, the oil phase at the top and the emulsion layer being intermediate. Ten graduated cylinders were used for each invert emulsion in each experiment, and stability was daily measured during 7 weeks following the preparation of emulsions.

Viability of the introduced bacterium and fungus into emulsions was determined by taking samples from invert emulsions stored at 25 ± 2° C at

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[b] 1 % wax (Radiasurf 7417) : polyethyleneglycol-PEG-1500 mono-stearate, water-soluble wax up to 1%, produced by FINA Chemicals, division of PETROFINA s. a., Nijverheidsstraat 52, rue de l'Industrie, 1040 Bruxelles-BELGIUM.

[c] 5% emulsifier (Radiasurf 7155) : Sorbitan mono-oleate, unsaturated emulsifier, produced by FINA Chemicals.

[d] 10% glycerine (Glycerin 4813) : 99.5% 1,2,3-Propanetriol, produced by FINA Chemicals.

[e] 95% oil (Radia 7108) : glycerol tricaprylate-caprate, produced by FINA Chemicals.

[f] 5% emulsifier (Span 20) : Sorbitan mono-laurate, produced by ICI Surfactants, registered in England no 1003645, London.

[g] 95% oil (Paraffin Vel 8577) : produced by VEL s. a., 3001-Leuven- BELGIUM.

weekly intervals after homogenization of the ingredients by shaking. Samples of 1 ml each was taken to make serial dilutions of 10^{-1} then subsamples of 100 µl of the last dilution (10^{-4}) were either spread on the surface of PDA plates or deposited on glass-slides kept in Petri-dishes under humid conditions. Ten Petri-dishes or glass-slides were used per weekly sample for each emulsion or control. The evaluation of the viability was made by determining the number of colony-forming-units (CFU) per ml of invert emulsion containing the bacterium or the percentage of conidial germination after 24 hours of incubation at 25 ± 2° C.

The water content of invert emulsions was determined after 24 hours of application under 3 levels of relative humidity (R.H.). These levels were controlled by using saturated solutions of 3 mineral salts [15]: 45% R.H. at 550 g/L potassium nitrite (KNO_2), 66% R.H. at 680 g/L sodium nitrite ($NaNO_2$), and 81% R.H. at 525 g/L ammonium sulfate [$(NH_4)_2SO_4$]. A fourth level of 100% R.H. controlled by water saturation was added. The technique invloved in this study was consisted of depositing 3 droplets (25 µl each) of invert emulsion per glass-slide held on small curved galss-rod and placed inside a closed desiccator containing saturated solutions of mineral salts prepared as indicated above. Seven glass-slides were used for each level of relative humidity and for each invert emulsion. By calculating the quantity of water lost during 24 hours after droplets' deposition, the percentage of lost water and remaining water could be determined.

A biological test was developed to evaluate efficacy of the antagonistic strains. The inoculation technique in this test was consisted of depositing at first, 25 µl droplets of microorganism suspension alone or with its formulation (concent. =10^8 conidia /ml for *Trichoderma* or 5X10^7 CFU / ml of *Bacillus*) on 20 mm-diameter green foliar discs of strawberry after inducing a small superficial wound at the disc center. Then , after 24 hours of antagonist application, depositing 25µl droplets of pathogen conidial suspension (concent.= 10^8 conidia / ml) on the same site of foliar discs inoculated previously with the antagonist. Incubation of foliar discs (treated and non-treated with antagonist) was made at 23± 2° C and 16 hours of illumination. The evaluation technique in this test was based on measuring the diameter of Botrytis typical lesions appeared on treated and non-treated

discs with the antagonist after 4 days of pathogen inoculation. Therefore, ratio of lesion diameter on treated and non-treated discs could be calculated to be used in the comparison . This evaluation was also based on measuring the relative density of Botrytis conidiophores on the infected surface of treated and non-treated discs after 8 days of pathogen inoculation.

Results

Prepared invert emulsions (IE.1 and IE.3) were regularly observed in order to determine their stability, viscosity, compatibility with introduced antagonistic microorganisms and their water content after 24 hours of application (Tables 1,2,3, and 4). The stability of these emulsions was checked regularly over a period of 7 weeks (Table 1). Results indicate that these fromulations have shown an emulsification rate of 99 to 100% after mixing the ingredients at weekly intervals, a separation rate of the two phases ranging from 0 to 1%.The viscosity of the same emulsions was also checked over a period of 7 weeks (Table 1). Results indicate that this viscosity is shown to be constant with 25 and 35 centipoises for the invert emulsions IE.1 and IE.3 respectively. Confirmation of these results was made by repeating the preparations and measurements for 5 times.

The number of colony-forming-units (CFU) per ml of bacterial formulation or the percentage of conidial germination of fungus formulation over the time after introduction are presented in Tables 2 and 3. After a repeated introduction of the microorganisms for 5 times, results indicate that there was no significant differences over the time between values of conidial germination percentage of *Trichoderma* when introduced into the invert emulsion IE.1 (Table 2). However, these values decreased significantly over the time when introduction occured into the invert emulsion IE.3 (Table 2). Similar results were obtained with number of CFU per ml of the bacterium when introduced into IE. 1 and IE. 3 respectively (Table 3). In addition, the formulation of the fungus using invert emulsions inhibited its germination during the whole period of observation in comparison with the non-formulated control where abundant germination of conidia and subsequent formation of mycelium was noticed within 1 to 2 weeks following suspension preparation (Table 2). Moreover, the formulation of the

bacterium using invert emulsions especially IE. 1 preserved its viability where the decrease was not significant in comparison with the significant decrease in viability in all non-formulated control treatments (Table 3).

The quantity of water retained in the invert emulsions after 24 hours of application was determined at different levels of relative humidity (table 4). The amount of water decreased significantly when relative humidity decreased from 100 to 45% for both invert emulsions. This content was amounted at 12.7% for IE.1 and 14.7% for IE.3 at 45% RH., instead of 34.6% for IE.1 and 18.6% for IE.3 at 81% R.H. Therefore, a sufficient quantity of water was retained in the two emulsions especially under dry conditions (50 to 80 %R.H.). This quantity is necessary for the survival of introduced microorganisms during 24 hours after application. These results were confirmed by repeating the experiment 3 times.

In addition to the above characteristics of our invert emulsions, biological efficacy of the strains of antagonistic microorganisms was studied when these microorganisms were formulated in invert emulsions compared with the non-formulated forms (Table 5). The mean diameter of Botrytis lesion on treated strawberry foliar discs with the antagonist compared with the non-treated discs with the antagonist (MDB CC) increased significantly in case of using both antagonists in non-formulated form at the different relative humidities (Table 5). Therefore, biological efficacy of the antagonists in non-formulated form reduced significantly at low relative humidities (especially at 66%). However, MDBLCC did not significantly increase in case of using the antagonists in formulated form at the same relative humidities (Table 5). Therefore, the formulation of both antagonists resulted in the same level of biological efficacy. However, at low relative humidities (especially 66%), this level was significantly higher than the level when these antagonists are not formulated. The mean relative density of Botrytis conidiophores on treated foliar discs with the antagonist compared with the non-treated discs with the antagonist (MRDBC) was nil

Table 1. Stability and viscosity of selected invert emulsions.

Characteristics of the emulsion	Time in days (d) or hours(h) after preparation	Formulation of the emulsion	
		IE.1: 50% aqueous Phase+50% oil phase	IE.3: 50% aqueous phase+50%oil phase
Emulsification rate of the two phases after preparation	1d	100%	100%
	14d	100%	100%
	28d	99%	99%
	42d	99%	99%
	49d	99%	99%
Separation of the two phases	1d	N.S	N.S
	14d	N.S	N.S
	28d	1% AP	1% AP
	42d	1%AP	1% AP
	49d	1%Ap	1%AP
Viscosity in Centipoises at room temperature (25±2°C)	1d	27.5	32.5
	14d	25	35
	28d	25	35
	42d	25	35
	49d	25	35

- Emulsification rate was measured by putting the invert emulsions in 100 ml graduated cylinders. Ten graduated cylinders were used for each invert emulsion in each experiment.
- N. S : No separation of the two phases composing the emulsion.
- AP : Aqueous phase which was seen separated from other ingredients.

Table 2: Viability of *Trichoderma* sp. (strain C1306) after introduction into the selected invert emulsions stored at 25±2°C

Time after preparation of invert emulsions and introduction of the fungus(in weeks)	Mean percentage of conidial germination of the fungus within 24 hours [t]			
	Invert emulsion (IE) containing the fungus		Control (fungal suspension only)	
	IE.1+fungus	IE.3+fungus	Blanco Control [w]	True Control [x]
0 [u]	64.6 [v] a	53.4 [v] a	67.8	85.6
1	61.2 a	51.0 a	C.G. [y]	C. G. [y]
2	58.2 a	48.2 ab	M. F. [z]	M. F. [z]
3	55.4 a	41.2 c		
4	54.2 a	40.0 c		
5	53.4 a	36.6 c		
6	53.0 a	31.0 d		

t. Five replicates were used for each percentage of conidial germination.

u. Conidial germination in this treatment was checked at the first day after introduction of the fungus.

v. Means within each column followed by the same letter for each invert emulsion are not significantly different ($p<0.05$) according to Duncan's multiple range test based on ANOVA table for the completely randomized design (CRD).

w. Blanco control is true control after passing in the mixer at 9th speed for 1 minute.

x. True control is the titrated fungal suspension without passing in mixer.

y. C.G: conidial germination observed in the suspension.

z. M.F:mycelial formation observed in the suspension.

Table 3. Viability of *Bacillus amyloliquefaciens* (strain GA1) after introduction into the selected invert emulsions and stored at 22±2°C.

Time after preparation of invert emulsions and introduction of the bacteria(in weeks)	Mean number of colony-forming-units (CFU) × 10^6 of bacteria per ml in weekly sampled portions [v]			
	Invert emulsion (IE) containing the bacteria		Control (bacterial suspension only)	
	IE.1+bacteria	IE.3+bacteria	Blanco Control [w]	True Control [x]
0 [y]	16.57 [z] a	13.25 [z] a	18.67 [z] a	22.52 [z] a
1	15.57 a	12.80 a	17.37 b	21.82 a
2	15.15 a	11.20 b	15.82 c	21.02 ab
3	14.92 a	10.80 b	15.07 c	20.00 bc
4	14.15 a	9.52 c	14.35 cd	19.10 cd
5	13.22 a	9.10 c	14.05 d	18.47 de
6	13.02 a	7.90 d	13.55 d	18.07 e

[v] Four replicates were used for each mean number of CFU×10^6 per ml. Each replicate-value was calculated from the counted number of colonies per perti-dish after spreading of 100 µl of bacterial suspension.

[w] Blanco control is true control passing in the mixer at 9th speed for 1 minute

[x] True control is the treated bacterial suspension (sporulating form) without passing in mixer and prepared directly from bacterial culture.

[y] Mean number of CFU×10^6 per ml in this treatment was checked in the first day after introduction of bacteria.

[z] Means within each column followed by the same letter for each invert emulsion or control are not significantly different ($p<0.05$) according to Duncan's multiple range test based on ANOVA table for the completely randomized design (CRD).

Table 4. Water content of selected invert emulsions after 24 hours of application at different relative humidities (temprature: 25±2°C).

Percentage of atomospheric relative humidity [x]	Mean percentage of water content in the invert emulsions (IE) after 24 hours[y]	
	IE.1	IE.3
100	82.2[z] a	85.1[z] a
81	34.6 b	18.6 b
66	20.4 c	16.8 b
45	12.7 c	14.7 b

[x] Controlled by using tightly closed desiccators containing saturated solutions of mineral salts.

[y] Three droplets of each invert emulsion (25 µl each) per one glass-slide . Seven glass-slides were used for each relative humidity or invert emulsion .

[z] Means within each column followed by the same letter for each invert emulsion are not significantly different ($p<0.05$) according to Duncan's multiple range test based on ANOVA table for the completely randomized design (CRD).

in both formulated and non-formulated forms of *Trichoderma* at all relative humidities (Table 5). However, MRDBC was higher (1-49 conidiophores) in both formulated and non-formulated form of *Bacillus* at all relative humidities (Table 5). Therefore, the formulation of both antagonists resulted in the same level of biological efficacy in terms of their effect on formation of Botrytis conidiophores. These results were confirmed by repeating the test 5 times using 12 replicates in each treatment or relative humidity.

Discussion

The strains of microorganisms involved in this study (GA1) of *Bacillus amyloliquefaciens* and C1306 of *Trichoderma* sp. , proved to be effective antagonists against *B.cinerea* (strain B20) on strawberry foliar discs in both formulated and non-formulated forms. These strains reduced the lesion development of *B.cinerea* growth and inhibited its ability to form

Table 5: Biological efficacy of 2 microorganisms antagonistic to *Botrytis cinerea* on foliar discs of strawberry (23±2 °C & different R.H.)

Relative humidity percentage during 48h. in desiccators	Application form of antagonistic microorganisms	Evaluation criteria of antagonistic effect			
		MDBLCC [v]		MRDBC [w]	
		Trichoderma sp. (CI306)	*Bacillus amyloliquefaciens* (GA1)	*Trichoderma* sp. (CI306)	*Bacillus amyloliquefaciens* (GA1)
100% R.H.	Non-formulated	0.20 c[z]	o.30 c[z]	o/+++	+/+++
	Formulated IE.1 [x]	0.44 b	0.58 b	o/+++	+/+++
	Formulated IE.3 [y]	0.52 b	0.61 b	o/+++	+/+++
81% R.H.	Non-formulated	0.62 b	0.69 ab	o/++	+/++
	Formulated IE.1 [x]	0.45 b	0.53 b	o/++	+/++
	Formulated IE.3 [y]	0.55 b	0.56 b	o/++	+/++
66% R.H.	Non-formulated	0.80 a	0.88 a	o/++	+/++
	Formulated IE.1 [x]	0.44 b	0.54 b	o/++	+/++
	Formulated IE.3 [y]	o.53 b	0.60 b	o/++	+/++

[v] MDBLCC indicates the mean diameter (in mm) of Botrytis lesion on treated foliar discs compared with the control (12 replicates per treatment).

[w] MRDBC indicates the mean relative density of Botrytis conidiophores on treated versus non-treated foliar discs with antagonists (12 replicates per treatment). Scale used : 0=no conidiophores, + = 1 - 49 , ++ = 50 - 199 , +++=>200 conidiophores.

[x and y] Ingredients of IE.1 and IE.3 indicated previously in materials & methods.

[z] Means within each column followed by the same letter for each antagonistic microorganism are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test based on ANOVA table for the completely randomized design (CRD).

conidiophores (Table 5). The antagonistic effect of several microorganisms was recently reported by many investigators: *Erwinia* sp. against *B.cinerea* on apples [3]; *Bacillus polymyxa* & *B.subtilis* against *B.cinerea* and *Pythium* spp. on peas & dwarf french beans [7]; *Bacillus pumilus* and *B. amyloliquefaciens* against *B.cinerea* on pears at postharvest stage [8]; *Trichoderma* spp. against *B.cinerea* on grape under field conditions [6]; and *Candida sake* against *B.cinerea*, *Penicillium expansum* Link and *Rhizopus nigricans* Ehrenb on apples [5]. However, no attempt was made by the above investigators to formulate any of the above antagonistic microorganisms. Our comparison of biological efficacy of the two strains of microorganisms antagonistic to *B.cinerea* in formulated and non-formulated form resulted in significant differences between these strains at different relative humidities (Table 5). In these comparisons, we used a novel criterium other than the usual one concerning biocontrol of *B.cinerea* according to the capacity of the antagonist to reduce the rate of pathogen sporulation [16]. Our novel criterium is based on measuring the diameter of Botrytis typical lesion on treated and non-treated foliar discs of strawberry with the antagonists since we have succeeded in producing typical lesions of *Botrytis* on living, green foliar discs of strawberry with the aid of small superficial wound and an inoculation with a concentration of 108 conidia / ml then incubation under humid conditions at 23 ± 2o C. The diameter of these lesions produced after application of the antagonist was used in comparing the efficacy of these microorganisms. This technique of production of *Botrytis* infection and using the lesion diameter for comparing the biological efficacy of antagonists is a something new in our work in comparison with the usual technique of evaluation of *Botrytis* biocontrol by suppression of its sporulation [16-18]. The significant differences obtained in biological efficacy of both antagonists *(Trichoderma* and *Bacillus)* in formulated or non-formulated forms at low relative humidities (Table 5), proved the capacity of both emulsions (IE.1 and IE.3) to retain enough quantity of water for survival of these antagonists. However, this efficacy was greatly reduced in case of using antagonists without formulation due to the loss of great quantity of water under dry conditions. (Table 5). Overall, results coincide with those of many workers on mycoherbicides using invert emulsion [9-11, 13, 19, 20].

The developed invert emulsions (IE.1 & IE.3) contained oils with moderate to low viscosity (28 centipoises for plant oil: Radia 7108 ad 124 centipoises for the mineral-oil: paraffin Vel 8577) and the unsaturated emulsifiers (Radiasurf 7155=sorbitan mono-oleate, and span 20=sorbitan mono-laurate), in addition to using a good mixing technique with a high speed during preparation. This explains the high stability and low viscosity of our invert emulsions indicated in Table 1. This explanation coincides with the conclusions of Connick et al., 1991 [13] and Daigle et al., 1990 [11] which indicate that stability and viscosity of invert emulsions used in mycoherbicide formulation depend on the type and viscosity of oil entering in emulsion composition, the type of emulsifier and mixing technique and its speed.

The good compatibility of invert emulsion IE.1 with introduced microorganisms is attributed to the ingredients entering in the formulation of this invert emulsion especially the oil (Radia 7108) and the emulsifier (Radiasurf 7155) which have shown to be much less toxic to introduced microorganisms than those entering in the formulation of IE.3 especially the oil (Paraffin Vel 8577) and the emulsifier (span 20). The difference in toxicity to introduced microorganisms is essentially related to the origin of oil entering in the formulation of each invert emulsion where it is of mineral-origin for Paraffin Vel 8577 and of plant-origin for Radia 7108, but in all cases the retained invert emulsions prepared from these oils are always having lower viscosity than those of oils (25 and 35 centipoises for IE.1 and IE.3 respectively). Another advantage for using the oil Radia 7108 and the emulsifier Radiasurf 7155 is that they are already accepted in the additivation of human foodstuffs in Belgium, and there is no problem of toxic residues during application. Until now, diffferent formulations of invert emulsion were used as carriers of weed-infecting fungi or mycoherbicides [9-13, 19, 20]. The viability of these fungi in the invert emulsions are not widely studied compared with our work (table 3), a single study was conducted by Daigle et al., 1990 [11] on the conidial germination of *Alternaria cassiae* (biocontrol agent of sicklepod: *Cassiae obtusifolia*) in a series of invert emulsions, they concluded that a minimum of 10% water content in the invert emulsion was necessary for obtaining a significant

germination of the fungus without reporting any value on conidial germination.

In the present formulations of invert emulsion, the addition of water-soluble wax (1% of aqueous phase) increased considerably its stability in comparison with the formulations containing no wax and the incorporation of glycerin 4813 (10% of aqueous phase) in our formulations of invert emulsion contributed to retaining more water in the formulation due to its action as depressor of water activity as a result of reduction of water droplets' surface area being in direct contact with the air and available to evaporation. Therefore, the quantity of retained water in our formulation is much higher than that reported by other authors working on improved mycoherbicides (10% of the invert emulsion developed by Daigle et al., 1990 [11]).

In conclusion, the admission of major ingredients of our invert emulsions specially IE.1 in the direct additivation of human foodstuffs and the distinctive characteristics of the emulsion such as high stability, low viscosity and no toxicity to introduced microorganisms would allow us to use this emulsion as carrier of other antagonistic microorganisms in biological control of phytopathogens. Therefore, the next step in our research project is to test the compatibility of these formulations with wide range of antagonists especially those used in postharvest biocontrol, then testing biological efficacy of these antagonists against postharvest pathogens.

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