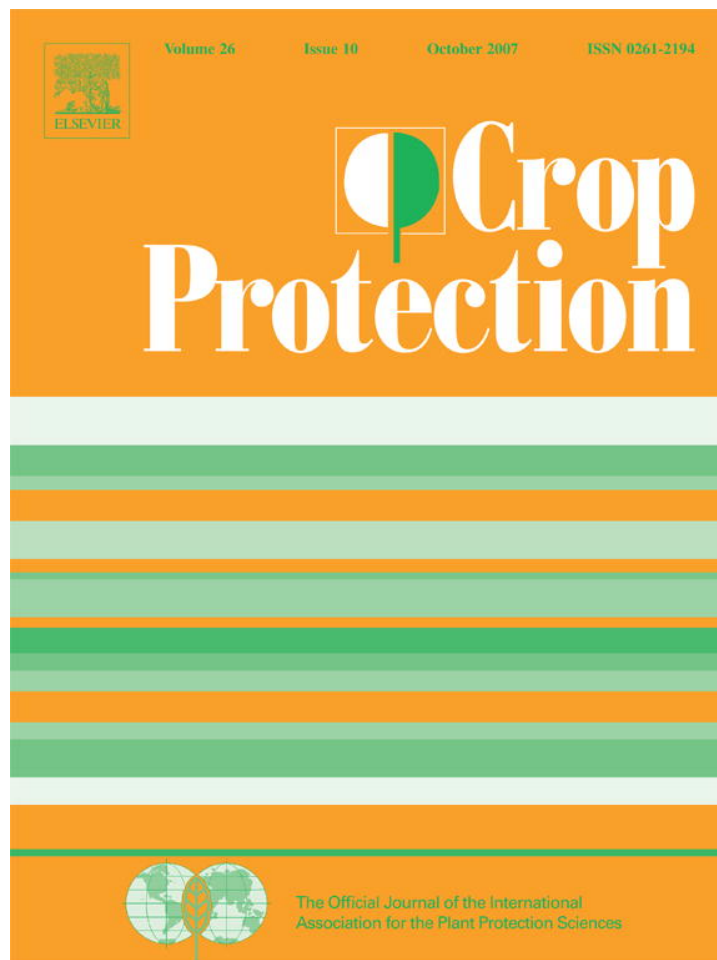


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Efficacy of alcohol alkoxyolate surfactants differing in the molecular structure of the hydrophilic portion to control *Phytophthora nicotianae* in tomato substrate culture

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Abstract

Phytophthora nicotianae is a common and destructive pathogen of numerous ornamental, agronomic and horticultural crops such as tomato, tobacco and citrus. Three monobranched C₁₃ alcohol alkoxyolate non-ionic surfactants were evaluated for *in vitro* inhibitory activity against the different asexual structures of *P. nicotianae*. The same surfactants, labelled MBA1301, MBA1303 and MBA1306, were tested for their *in vivo* control capacity against *P. nicotianae* root rot of tomato (*Lycopersicon esculentum*) under glasshouse conditions. MBA1301, MBA1303 and MBA1306 differ in the molecular structure of the hydrophilic portion. The molecular weight of MBA1301 is comparable to that of MBA1303 and is eight times lower than that of MBA1306. The main *in vitro* activity for MBA1301 and MBA1303 was a direct lytic effect on the zoospores. Zoospore lysis was already observed in the presence of 1 µg ml⁻¹ of these two surfactants and almost no zoospores survived an addition of 5 µg ml⁻¹ surfactant. In addition, MBA1301 and MBA1303 reduced sporangia formation at a concentration of 5 µg ml⁻¹. Both surfactants only affect mycelium growth at concentrations as high as 100 µg ml⁻¹. MBA1306 did not show any effect on sporangia formation, zoospore release and mycelium growth of *P. nicotianae* at a concentration 10 times that of the other two surfactants. A good *in vivo* control of *P. nicotianae* on tomato in substrate culture was obtained for MBA1301 and MBA1303 whereas the control capacity of MBA1306 was significantly lower. The results of this research indicate that non-ionic alcohol alkoxyolate surfactants can be used to control tomato root rot caused by *P. nicotianae* in substrate culture. In addition, the size rather than the arrangement of the polymers in the hydrophilic portion of the surfactant molecule determines the efficacy to control tomato root rot caused by *P. nicotianae* in substrate culture.

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Keywords: *Phytophthora nicotianae*; Zoospore pathogens; Non-ionic surfactants; Tomato root rot; Substrate culture

1. Introduction

Under intensively managed agricultural soil-plant systems, the impact of certain pathogens has become much more important than in more natural, better balanced and growing systems. Hydroponic and substrate culture systems are commonly used in the greenhouse industry as an alternative to mitigate soil borne root pathogens, but root diseases continue to be a major problem. *Phytophthora nicotianae* Breda de Haan (= *P. parasitica* Dastur) is one

of the most widespread and destructive soil-borne pathogens affecting over 300 host species (Erwin and Ribeiro, 1996). Symptoms of the disease caused by this zoospore plant pathogen on tomato include buckeye, brown, and root rot, stem girdling, damping-off and foliar blight (Erwin and Ribeiro, 1996).

Early detection and immediate proper action, especially in re-circulating nutrient solutions of hydroponic cultures, are crucial for the implementation of efficient control strategies (Grote et al., 2002). However, control of the disease still relies on traditional pesticide formulations containing active ingredients such as mancozeb, maneb and others. More target-specific measures such as the use of the

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inhibitory properties that some non-ionic surfactants possess against zoosporic pathogens may improve the efficiency of control strategies in the future.

Several research groups have shown that non-ionic surfactants can exhibit a lytic activity against zoospores (Tomlinson and Faithfull, 1979, 1980; Stanghellini and Tomlinson, 1987; Stanghellini and Miller, 1997; Demeulenaere and Höfte, 2000). Stanghellini and Rasmussen (1994), and Stanghellini et al. (1996a, b, 2000) also proved that non-ionic surfactants can be used to control root-infecting zoosporic plant pathogens in hydroponic systems. In addition, De Jonghe et al. (2005a) tested the effectiveness of the alcohol alkoxyolate MBA1301 against *Phytophthora cryptogea* in the hydroponic forcing system of witloof chicory. Their results showed that two weekly applications of $10 \mu\text{g ml}^{-1}$ MBA1301 could largely control the pathogen in a semi-commercial hydroponic growing unit.

Surface-active agents, including non-ionic surfactants, are known to disrupt cell membranes because they dissolve the lipid membrane. This lowers the surface tension of the membrane, allowing water to flow into the cell and ultimately resulting in lysis. The balance between the hydrophilic and lipophilic sections of the molecules is essential for these processes (Gareth, 2000). Most literature about the structure–function relationship of antimicrobial surfactants, specifically in relation to the interaction with cell membranes, is human medicine related (Mason et al., 2006; Ouellet et al., 2006). The amphipatic structure allows surfactants to form polymeric micelles, which can incorporate an active ingredient and subsequently act as an efficient drug carrier through the membrane. The antimicrobial activity of the surfactant itself is usually related to the length of the fatty acid chain, a key factor that determines solubility of the molecule (Ingram and Buttke, 1984; Garg and Muller, 1993; Kubo et al., 1995; Lee et al., 1998; Malina and Shai, 2005).

In this paper, three non-ionic surfactants with differences in composition of the hydrophilic portion but with the same monobranched C_{13} lipophilic part were compared for their inhibitory activity against some asexual structures of *P. nicotianae*. In addition, the potential of these alcohol alkoxyolates to control *P. nicotianae* on tomato in substrate culture was evaluated.

2. Materials and methods

2.1. Plant material

Tomato (*Lycopersicon esculentum* Mill.) of the cultivar “Moneymaker” (Royal Sluis, Enkhuizen, The Netherlands) was sown in trays containing potting soil (Klassman no. 4) and seedlings were transferred to substrate blocks (Grodan BV, Roermond, The Netherlands) of $10 \times 10 \text{ cm}$ and 6 cm high at the second leaf stage. They were further grown in greenhouse conditions until the fourth leaf stage.

2.2. Pathogen

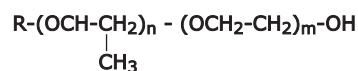
Phytophthora nicotianae strain no. PD97/91, isolated from tomato, was supplied by the “Plantenziektenkundige Dienst”, Wageningen, the Netherlands. Axenic cultures of the strain were grown in darkness at 24°C on V8 juice agar (V8A) (200 ml V8-juice (Campbell Foods Belgium, Puurs, Belgium), 15 g agar (Pulvis) (Federa, 1130 Brussels, Belgium), 2 g CaCO_3 and 800 ml distilled water).

2.3. Sporangia, zoospore production and inoculum preparation

Sporangia and zoospores were produced under aseptic conditions according to the procedure described by De Jonghe et al. (2005b). In *P. nicotianae*, asexual sporulation, the development of sporangia and subsequent zoospore release, were induced synchronously *in vitro* by replacing the V8 juice agar with a mineral salts solution and incubation under a continuous light regime. The method was used for the zoospore production of *Phytophthora cryptogea* and proved valid for sporangia and zoospore production of *Phytophthora nicotianae*.

2.4. Surfactants

The surfactants used in this study were Atplus MBA1301, Atplus MBA1303 and Atplus MBA1306, non-ionic alcohol alkoxyolates with general formulae presented in Fig. 1. The lipophilic portion of all three surfactants is identical and consists of a monobranched C_{13} chain with the branch on the second C-atom. The hydrophilic portion of MBA1301 consists of a block copolymer of ethylene oxide and propylene oxide molecules and has a total molecular mass comparable to that of MBA1303. The hydrophilic part of MBA1303 consists of a randomized polymer section. The hydrophilic part of MBA1306 also comprises a block copolymer structure with a molecular mass of about eight times higher than MBA1301 and MBA1303 (Fig. 1).



R = monobranched C_{13} -chain

m = degree of ethoxylation

n = degree of propoxylation

MBA1301 m+n = 8
Arrangement: block copolymer

MBA1303 m+n = 10
Arrangement: randomized polymer

MBA1306 m+n = 64
Arrangement: block copolymer

Fig. 1. Chemical structure of the alcohol alkoxyolates Atplus MBA1301, MBA1303 and MBA1306.

2.5. *In vitro* assays

Different concentrations (0, 10, 20, 100, 500 and 1000 $\mu\text{g ml}^{-1}$) MBA1301, MBA1303 or MBA1306 were amended in the V8 agar medium. The mycelium growth rate was tested by central inoculation of a mycelium plug (\varnothing 5 mm) on a Petri dish containing 15 ml of the medium. The growth was recorded 24, 48 and 72 h after inoculation as the average of four measurements (in each direction of two right-angled lines crossing the centre of the mycelium plug). Eight replications were performed for each concentration and the experiment was repeated once.

Different concentrations of surfactant (0, 1, 5, 10, 20 and 50 $\mu\text{g ml}^{-1}$) were added to the mineral solution with mycelium plugs (see Section 2.3) and the effects on sporangia formation and zoospore release were microscopically observed. Sporangia development was assessed on a 1–5 scale (no sporangia development, score 1; 25%, 50% and 75% of normal sporangia development, score 2, 3 and 4, respectively; normal sporangia development, score 5). Subsequently, zoospore release was induced by placing the Petri dishes at 4 °C for 1 h, followed by an accommodation period of 1 h at room temperature. Zoospore release was assessed by calculating the proportion empty sporangia on the total sporangia production and the obtained percentages were then classified: class 1, no zoospore release; class 2, up to 25%; class 3, 26–50%; class 4, 51–75% and class 5, 76–100% zoospore release. The experiment was performed three times.

Surfactant (0, 1, 5, 10, 20 and 50 $\mu\text{g ml}^{-1}$) was added directly to zoospore suspensions yielded from untreated sporangia to study zoospore lysis. Five minutes after surfactant application, the remaining zoospores were forced to encyst by centrifugation (5 min at 3500 rpm). The pellet of encysted zoospores was re-suspended in sterile distilled H_2O and counted in 10 replicates of $0.2 \times 0.2 \times 0.1$ mm volumes by means of a haematocytometer. The experiment was performed three times.

2.6. *In vivo* experiments

For each experiment, eight tomato plants for each surfactant/concentration combination and eight control plants were randomized and placed under greenhouse conditions with a 10 h photoperiod. The treatments were applied on top of the substrate cube, and concentrations were calculated on a total volume of 600 ml per plant. Where applicable, surfactant application was followed by an infection with 10^2 zoospores *P. nicotianae* ml^{-1} . In that case, pathogen and MBA treatments were administered separately in half of the total volume of nutrient solution (300 ml). Of all three alcohol alkoxyates (50 and 100 $\mu\text{g ml}^{-1}$) were applied to the nutrient solution to test for potential phytotoxicity and direct effects on the growth of tomato plants.

For the infection trials, 10, 20 or 50 μg surfactant per ml were added as a single application at the start of the

experiment and inoculated control treatments were included in the experimental set-up.

For all experiments, disease incidence (% diseased roots), disease severity (score system, summarized in Table 1), plant height and leaf stage were recorded 14 days after the start of the experiment. The phytotoxicity experiments were repeated once. The control assays of *P. nicotianae* were carried out three times.

2.7. Statistical data analysis

Statistical analysis of data was performed using the software package SPSS for Windows 12.0.1. (SPSSinc, Chicago, IL, USA). For disease severity data, the conditions of normality and homogeneity of variances were not fulfilled and a suitable transformation was not available. These data sets were submitted to the non-parametric Kruskal–Wallis and Mann–Whitney tests and α -values were corrected for multiple comparisons. Results in figures presented as multiple section bars were statistically treated as one data set, unless otherwise indicated in the figure legend. The plant height data were subjected to analysis of variance (ANOVA). Means were separated using Tukey's honestly significant difference test, a post hoc test for multiple comparisons.

3. Results

3.1. *In vitro* experiments

3.1.1. Influence of the surfactants on the mycelial growth rate

The effect of the three alcohol alkoxyates on the mycelial growth of *P. nicotianae* on V8 agar is shown in Fig. 2. No effects could be observed for MBA1306 up to the highest concentration tested (1000 $\mu\text{g ml}^{-1}$). For both MBA1301 and MBA1303, a significant reduction of the

Table 1
Evaluation of tomato root rot symptoms following an infection with *P. nicotianae* zoospores

Score	Class	Disease symptoms
1	Healthy plant	Healthy plant, no symptoms.
2		Occasional brown root tips or zones.
3		Clear <i>P. nicotianae</i> symptoms, frequent brown moderate to large lesions on the roots. No symptoms on stem (base) present.
4	Root rot symptoms	Heavily diseased plants, poorly developed root system with excessive rotting symptoms. Symptoms on stem and wilting are possible.
5	Collapsed plants	Collapsed plants, due to narrowing of the stem base.

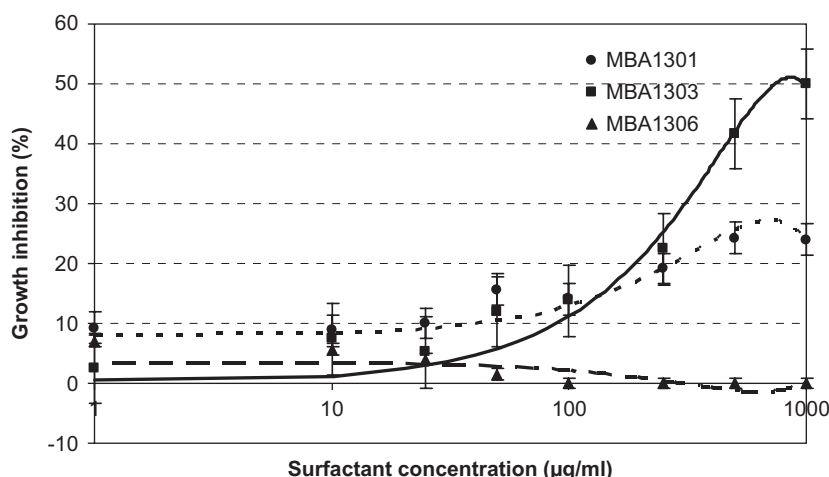


Fig. 2. Growth inhibition (%) compared to the control after 72 h caused by different concentrations MBA1301, MBA1303 or MBA1306 amended in the growth medium (V8A) when compared to the average normal mycelial growth of *P. nicotianae* (untreated control, 32.1 ± 1.8 mm). For clarity, the values in the x-axis are presented in a log scale. Error bars indicate the standard deviation.

growth rate could be observed for $100 \mu\text{g ml}^{-1}$ surfactant and more.

3.1.2. Influence of the surfactants on sporangia development and zoospore release

Strong inhibitory effects on sporangia development and zoospore release were recorded when mycelium plugs of *P. nicotianae* were exposed to MBA1301 or MBA1303, whereas MBA1306 was not effective at any of the tested concentrations (Fig. 3). In general, reduction of sporangia formation started in the presence of at least $5 \mu\text{g}$ MBA1301 or $10 \mu\text{g}$ MBA1303 ml^{-1} and good control of these important pathogen structures with complete absence of sporangia development in at least 75% of the replicates was obtained for $20 \mu\text{g}$ surfactant and more. In addition, Fig. 4 shows that a minimum of $5 \mu\text{g ml}^{-1}$ MBA1301 or $10 \mu\text{g ml}^{-1}$ MBA1303 was enough to reduce zoospore release significantly. Again, no effect of MBA1306 was recorded at any of the tested concentrations.

3.1.3. Influence of the surfactants on the zoospore survival

Both MBA1301 and MBA1303 already had a significant effect on zoospore survival at a concentration of $1 \mu\text{g}$ surfactant ml^{-1} mineral solution (Fig. 5). From $10 \mu\text{g ml}^{-1}$ onwards, MBA1301 and MBA1303 almost completely eliminated the presence of zoospores in the mineral solution. At $5 \mu\text{g ml}^{-1}$ MBA1306 also showed a significant lytic effect, but even at $50 \mu\text{g ml}^{-1}$ more than 30% of the zoospores survived.

3.2. Phytotoxicity test

No phytotoxic effect was observed for both concentrations and for neither one of the non-ionic alcohol alkoxylates. Moreover, growth-promoting effects, were recorded for MBA1301 and MBA1303. Control plants had an average height of 22.36 cm, whereas the height of

plants treated with a concentration of $50 \mu\text{g ml}^{-1}$ MBA1301, MBA1303 or MBA1306 was 25.10, 25.56 and 24.34 cm, respectively. Fig. 6 presents the same data as percentage relative to the control.

3.3. Control of *P. nicotianae* in substrate culture

An infection with 10^2 zoospores of *P. nicotianae* resulted in more than 85% of plants with root rot symptoms in the inoculated control plants. Moreover, most of those plants had collapsed due to collar rot. No plants with root rot symptoms were observed in the uninfected control treatment (Fig. 7).

A minimum concentration of $50 \mu\text{g ml}^{-1}$ MBA1301 or MBA1303 added to the nutrient solution was able to control the disease. Both alcohol alkoxylates reduced the number of plants with *P. nicotianae* symptoms to less than 30%. MBA1306 did not significantly reduce tomato root rot incidence at a concentration of $50 \mu\text{g ml}^{-1}$, leaving over half of the plants with *P. nicotianae* symptoms (Fig. 7).

4. Discussion

Several studies have already clearly demonstrated that non-ionic surfactants can be used to effectively control zoospore plant pathogens in soil-free growing systems (Stanghellini et al., 1996a, b; De Jonghe et al., 2005a).

This study shows that the non-ionic alcohol alkoxylate MBA1301, which proved effective in the control of brown root rot caused by *Phytophthora cryptogea* in the hydroponic forcing of witloof chicory (De Jonghe et al., 2005a), also effectively controls *Phytophthora nicotianae* root and collar rot of tomato. Comparison of the control capacity of MBA1301 with two other alcohol alkoxylates revealed that the size rather than the arrangement of the polymers in the hydrophilic portion of the surfactant plays an important

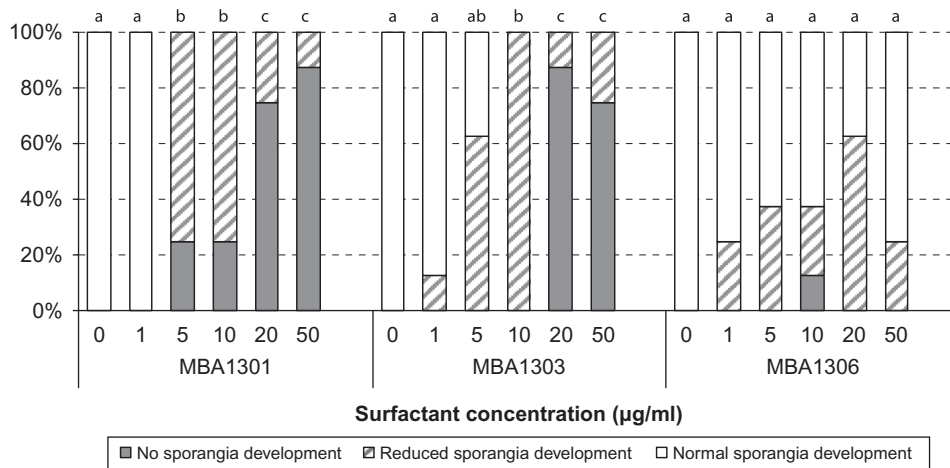


Fig. 3. Comparison of the influence of 1, 5, 10, 20 and 50 µg ml⁻¹ MBA1301, MBA1303 or MBA1306 on the sporangia development of *Phytophthora nicotianae*. Columns marked by the same letter are not significantly different at the 0.05 level determined by the Mann–Whitney test.

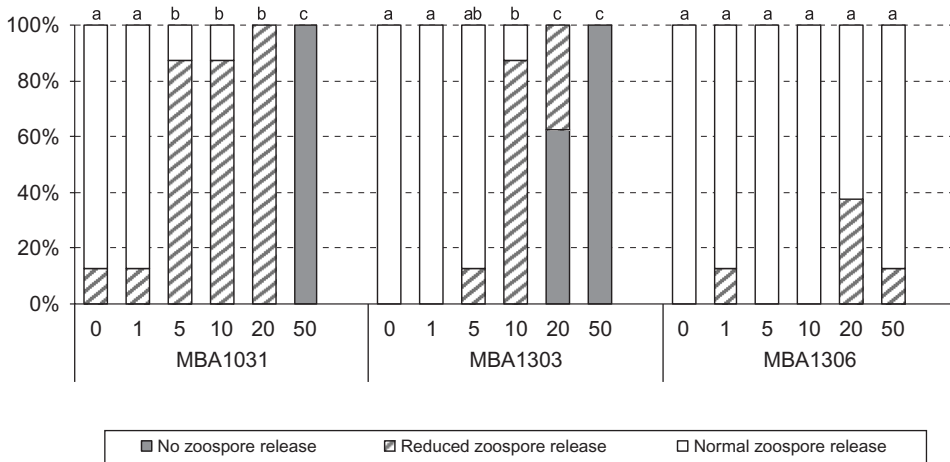


Fig. 4. Comparison of the influence of 1, 5, 10, 20 and 50 µg ml⁻¹ MBA1301, MBA1303 or MBA1306 on the release of *Phytophthora nicotianae* zoospores. Columns marked by the same letter are not significantly different at the 0.05 level determined by the Mann–Whitney test.

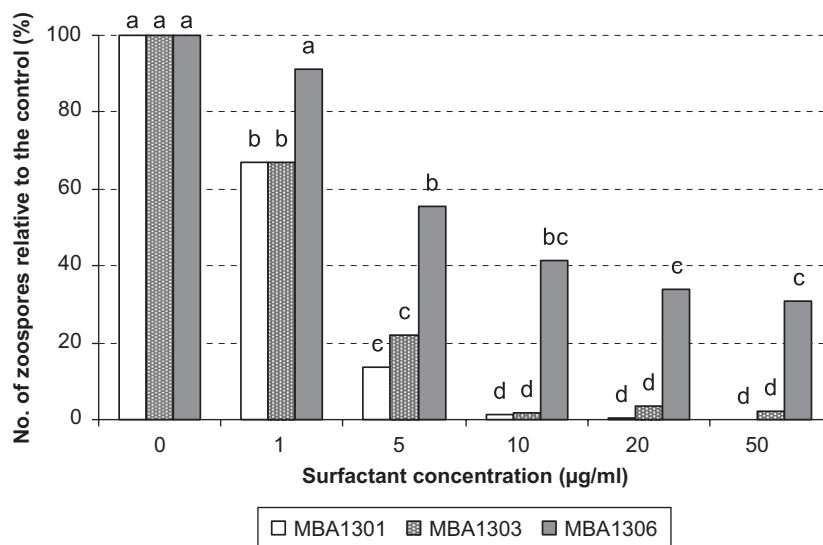


Fig. 5. Comparison of the influence of 1, 5, 10, 20 and 50 µg ml⁻¹ MBA1301, MBA1303 or MBA1306 on the zoospore lysis of *Phytophthora nicotianae*. Columns marked by the same letter are not significantly different at the 0.05 level determined by the Mann–Whitney test.

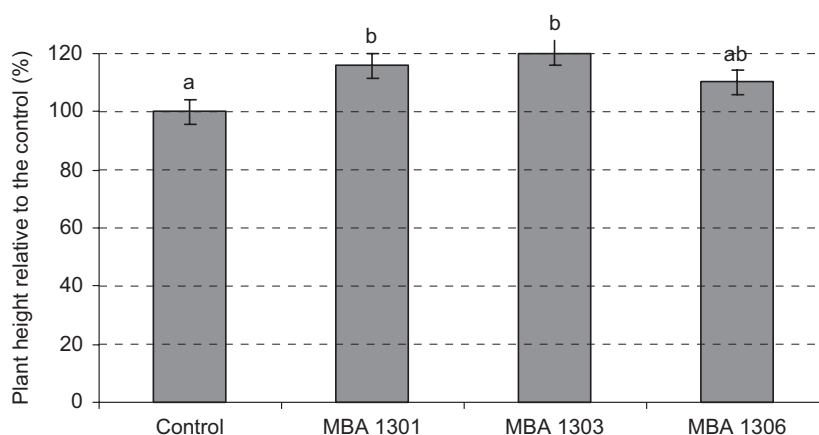


Fig. 6. Average tomato plant height relative to the control (% ± standard error), 14 days after treating uninoculated plantlets with 50 µg MBA1301, MBA1303 or MBA1306 per ml nutrient solution. Columns marked by the same letter are not significantly different at the 0.05 level determined by the Tukey's honestly significant difference test.

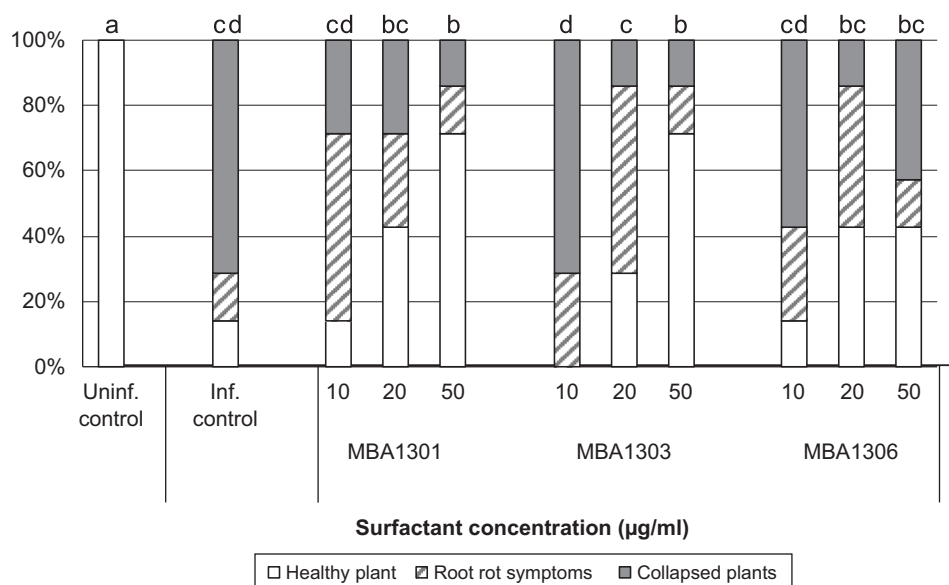


Fig. 7. Comparison of the influence of 10, 20 or 50 µg MBA1301, MBA1303 or MBA1306 per ml nutrient solution on the disease incidence and severity of *Phytophthora nicotianae* on tomato substrate culture. Columns marked by the same letter are not significantly different at the 0.05 level determined by the Mann-Whitney test.

role in its ability to lyse the zoospores, and thus influences its efficiency in controlling the pathogen.

In general, from the *in vitro* experiments, it can be concluded that mycelium is the most resistant structure, followed by sporangia and zoospores. The high sensitivity of the zoospores may be explained by the absence of a protective cell wall. This was also found by Oros et al. (1999) who tested the direct effect of 11 sulfosuccinic acid ester surfactants against the asexual spores of *Plasmopara halstedii*. In their research, it was established that the plasma membrane of the cell wall-lacking zoospores showed the highest and the resting zoosporangia the lowest average sensitivity towards the surfactants. Similar responses were found for the antibiotic 2,4 diacetylphloroglucinol (2,4 DAPG) on the asexual structures of *Pythium* spp. (de Souza et al., 2003).

Both MBA1301, with a block copolymer arrangement, and MBA1303, with a randomized polymer section of the hydrophilic portion, but comparable in molecular mass, had a similar effect on mycelium, sporangia development, zoospore release and lysis. Oros et al. (1999) showed that both strength and selectivity of the biological activity of anionic sulfosuccinic acid ester surfactants mainly depends on the lipophilicity of the molecule. As far as we know, there is no record that links the arrangement of the polymer section to the antifungal activity of the component. Most related literature deals with the design and synthesis of (block co) polymers with as its aim the solubilisation of a membrane active molecule, such as amphotericin B (Kwon and Kataoka, 1995; Yu et al., 1998).

The effect of MBA1306 on the asexual structures of *P. nicotianae* was limited, compared to that of the other two

non-ionic surfactants. Since the three tested surfactants comprise the same lipophylic monobranched C13 section, the large hydrophilic portion of MBA1306, with a molecular mass eight times as high as that of MBA1301 and MBA1303, is most likely the reason for this lower effect. Possibly, the molecule is less able to interact with the zoospore membrane.

Similar results were obtained in the *in vivo* experiments. MBA1301 and MBA1303 have a comparable good disease-suppressing effect following an artificial infection of *P. nicotianae* on tomato plants in substrate culture. Yet no efficient disease control was obtained for MBA1306. The effective concentration of $50 \mu\text{g ml}^{-1}$ for MBA1301 and MBA1303 is higher than what was necessary to control *Phytophthora cryptogea* on witloof chicory in hydroponic culture (De Jonghe et al., 2005a). Because of the fast biodegradability of the surfactants, other research groups also pointed out the need for repeated surfactant applications. In that respect, Stanghellini et al. (1996a) reached a satisfactory disease control of *P. capsici* on peppers in a soil-free growing system with weekly repeated applications of $20 \mu\text{g ml}^{-1}$ of the non-ionic surfactant they used in their study. De Jonghe et al. (2005a) showed that two weekly applications of $10 \mu\text{g ml}^{-1}$ were enough to obtain a satisfactory disease control of brown root rot in witloof chicory. So, the initial surfactant concentration to control *Phytophthora* root rot of tomato by MBA1301 or MBA1303 needs to be higher, but no repetition of the treatment was necessary. In substrate-grown greenhouse tomatoes, *P. nicotianae* mainly causes root and stem-base rot on young plants; plants are able to overcome the presence of *P. nicotianae* inoculum in the nutrient solution at later growth stages. Buckeye fruit rot is seldom observed because there is no contact between fruits and substrate.

Stanghellini et al. (2000) also effectively used non-ionic surfactants to control *P. capsici* on pepper, both in top- and sub-irrigation systems, and in organic substrate. Unfortunately, the results of preliminary experiments to control *P. nicotianae*, by means of the alcohol alkoxylates used in this study, on tomato plants grown in potting soil were extremely variable (data not shown). This may be partly due to adsorption of the surfactants in the potting soil, making them less available and increasing the minimum concentration needed to be effective. Since non-ionic and cationic surfactants have a much higher sorption on soil and sediment than anionic surfactants (Ying, 2006), it might be interesting to look for an anionic surface-active compound with anti-oomycete properties to use in soil conditions.

Finally, consistent with the higher yield and improved quality results that De Jonghe et al. (2005a) recorded on witloof chicory crops harvested from MBA1301-treated growing trays, improved growth of tomato plants was observed for the MBA1301 and MBA1303 treatments in the phytotoxicity experiments. Again, the lowest effect was observed for MBA1306. So far, no conclusive remarks can

be made to reveal the nature of the plant growth-promoting effect, and studies are still ongoing.

In conclusion, the alcohol alkoxylates MBA1301 and MBA1303 can be used as a single application of $50 \mu\text{g ml}^{-1}$ in the nutrient solution to protect tomato plants in rock wool slab culture from a subsequent infection with zoospores of *Phytophthora nicotianae*. The arrangement of the ethylene oxide and propylene oxide subunits of the hydrophilic part does not seem to play a role in the efficacy of the surfactant. Alcohol alkoxylates with a limited size of the hydrophilic portion of the amphipathic molecule have a higher inhibitory effect on the asexual structures of the pathogen than a structurally similar surfactant molecule with a large hydrophilic portion, and also provide a better *in vivo* control effect in substrate culture of tomato.

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