Control of blue mould and bitter rot of apples, and brown rot of peaches by *Citrus aurantium* extract-based product BIOLASTING®

M. Almazán[a], J.M. Lara and C. Fernández

R+D+i Department, FUTURECO BIOSCIENCE S.A., Av. del Cadí 19-23, Pol. Industrial Sant Pere Molanta, Olèrdola 08799, Barcelona, Spain.

Abstract

Reduction of postharvest fruit losses is a major agricultural goal affected by withdrawal of effective chemical products due to environmental or health concerns. Novel clean solutions are necessary. Activity of BIOLASTING®, a new cold pressed citrus extract-based formulation, was tested in vitro and in vivo against *Colletotrichum gloeosporioides* (bitter rot of apples), *Penicillium expansum* (blue mould of apples) and *Monilinia laxa* (brown rot of peaches). BIOLASTING® in vitro reduced the colony diameter of the three pathogens, but reduced spore production only of *C. gloeosporioides* and *M. laxa*. In controlled laboratory tests, 'Golden Delicious' apples were wound-inoculated with a spore suspension of *P. expansum* or *C. gloeosporioides,* while 'Amarillo Tardío' peaches were inoculated with spore solution of *M. laxa*. Two hours after inoculation with the corresponding pathogen, fruit were treated by immersion in the product solution for 2 min. Lesions diameter was measured at 26, 28 or 31 days after application according to the pathogen. In the *C. gloeosporioides* tests, quality parameters also were assessed (firmness, soluble solid content and titratable acidity). BIOLASTING® was also tested at semi-commercial conditions against *M. laxa* on peaches. The product was applied by drench or spray at two doses (1 and 1.5%). In the apples tests, BIOLASTING® applied at 1% reduced by 78% the area infected by *C. gloeosporioides* and improved all the assessed quality parameters, but was unable to control *P. expansum* at any doses. In peach laboratory tests, BIOLASTING® at 2 and 3% reduced *M. laxa* severity by 52 and 71%, respectively. Under semi-commercial conditions, BIOLASTING® exhibited more than 90% reduction of rot surface without significant differences between doses or type of application. The incidence was also reduced with better results (93%) at the 1.5% dose applied as drench. Based on these results, it can be concluded that postharvest applications of BIOLASTING® might significantly reduce bitter rot on apples and brown rot on peaches.

Keywords: *Colletotrichum gloeosporioides*, *Penicillium expansum*, *Monilinia laxa*, cold pressed citrus extract, postharvest diseases

INTRODUCTION

Postharvest diseases due to fungal infections cause high losses to fruits and vegetables during transport, storage and commercialization. There are many fungal diseases causing major fruit losses not only in terms of production but also in terms of quality. Brown rot is the main disease on stone fruit and *Monilinia laxa* is its most common causal agent in European and South African growing areas (Neri et al., 2007). Blue mould caused by *Penicillium expansum* is the most important postharvest disease of pome fruit worldwide, and bitter rot, caused by *Colletotrichum gloeosporioides*, is a destructive apple fruit rot in most of the countries where these fruit are grown. Although synthetic fungicides are quite efficient in the control of postharvest pathogens, the limitation of the authorized active ingredients, the increasing consumer concern for food chemical residues and the emergence

[a]E-mail: marta@futurecobioscience.com

of resistant pathogens to the most commonly used fungicides, have increased the efforts in investigating and developing new alternatives or complementary tools. Over the years, plant extracts have gained scientific interest for their antifungal activity against several postharvest diseases due principally to the presence of different classes of phenolic compounds (Rauha et al., 2000; Schena et al., 2008). BIOLASTING® (Futureco Bioscience S.A.) is a novel formulation consisting of 50% (w/w) cold pressed citrus-extract from *Citrus aurantium* (Sour orange). The principal active components of this product are organic acids and flavonoids, like naringin, hesperidin and quercetin, which have been reported as natural compounds with antimicrobial activity (Sanzani et al., 2009; Ortuño et al., 2006). Therefore, the objective of the present study was to evaluate the in vitro activity of BIOLASTING® against some of the major postharvest fungal pathogens. The potential of BIOLASTING® in preventing disease development was also tested on artificially inoculated fruits under laboratory and semi-commercial conditions.

MATERIALS AND METHODS

Fruit material, fungal cultures and products

'Golden Delicious' apples and 'Amarillo Tardío' peaches were obtained from a commercial packinghouse in Vilafranca del Penedès (Barcelona, Spain). Fruits were selected free of wounds and rots and as much as possible homogeneous in maturity and size, and stored at 4°C until needed. Conidia of *P. expansum* and *C. gloeosporioides* were obtained from 10-day-old potato dextrose agar (PDA) cultures incubated at 26°C in the dark. Conidia of *M. laxa* were collected from 10-day-old yeast-peach agar cultures (YPA, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 20% macerated peach, 20 g agar-agar) incubated at 25±2°C under a photoperiod of 16:8 h (light:dark). Inoculum was obtained by flooding petri plates containing the sporulated culture with sterile distilled water. Spore concentration was determined with a haemocytometer and adjusted at 10^6 spores mL^{-1}, for *P. expansum* and *M. laxa*, and 10^4 spores mL^{-1} for *C. gloeosporioides*. BIOLASTING® was used at different concentrations from 0.5 to 3% v/v, diluted in distilled water. Thiabendazole (TBDZ) 60% w/v, (Textar 60T, TECNIDEX S.A, Spain), or Fludioxinil 23% (w/v) (Scholar®, Syngenta, Switzerland) were used as reference products in the different assays.

In vitro inhibition of growth and spore production by *P. expansum, C. gloeosporioides* and *M. laxa*

To assess the effect of BIOLASTING® on the growth and spore production by the selected pathogens, agar plugs from the growing edge of 1-week-old cultures, were placed in the centre of PDA (*P. expansum* and *C. gloeosporioides* experiments) or YPA (*M. laxa*) plates supplemented with the tested product to reach a final concentration of 0.5, 1 and 2%. PDA or YPA plates amended with TBDZ at a final concentration of 1.2 ppm were also prepared as a chemical control. All products were added to warm culture media after autoclaving (20 min, 121°C). Treatments were set up in triplicate and repeated twice. Fungal growth was measured as colony diameter (mm) at 3, 5 and 10 days post-inoculation. Spore production was evaluated also at 10 days by flooding the plates with 20 mL of sterile distilled water. Spores were counted with a haemocytometer the same day.

Efficacy tests on fruits under laboratory conditions

Fruits were surface-disinfected by immersion for 2 min in a dilute solution of sodium hypochlorite (1% active chlorine), washed with tap water and left in a dry place to remove excess of water on the surface. Then, all fruits were wounded in the equatorial zone with a cork borer. Wounds (4×4×2 mm) were allowed to dry for 1 h and then inoculated with 50 µL of the pathogen. Two hours after inoculation with the corresponding pathogen, fruits were treated by immersion on the product solution for 2 min. A treatment with the reference product, TEXTAR 60T at the commercial dose, 0.2%, was included here. Peaches inoculated with *M. laxa* were maintained at 4°C during 26 days after application (DA-A) while apples were kept at room temperature (20±2°C) during 28 or 31 days for *C. gloeosporioides* and *P.*

expansum, respectively. After the storage period, disease severity, expressed as percentage of rot surface, was determined. For the *C. gloeosporioides*-apple tests, also quality parameters were assessed as firmness (FM), soluble solid content (SSC) and titratable acidity (TA).

Efficacy at semi-commercial trial under cold storage conditions against *Monilinia laxa* on peaches

During 2014 season, a semi-commercial trial was conducted in Alginet (Valencia, Spain). 'Amarillo Tardío' peaches were randomly chosen at harvest, disinfected with 2% sodium hypochlorite for 2 min, washed with tap water and air-dried. Peaches were wounded (5×3 mm) at the equator of each fruit with a sterile nail. When the wounds were air-dried, peaches were challenged with 20 µL of a conidial suspension of *M. laxa* (10^4 spores mL^{-1}). After inoculation, peaches were treated with Scholar® by drench at a commercial dose (0.25%), or with BIOLASTING® at two doses, 1 or 1.5% (v/V), either by drench (20 s) or spray. Non-treated fruit served as a control. Every treatment consisted of 4 plots with 100 fruits per plot. All fruit were stored at 0-0.5°C and 95-98% relative humidity (HR) during 30 days. Incidence and severity (% of rot surface) of brown rot in peaches was evaluated at 3, 7, 15, 21 and 30 DA-A of the treatments.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using R Software (version R i386 3.1.2). Least significant differences values (LSD; $P<0.05$) were used to compare the means. The in vitro experiments were repeated twice. Data from the spore production evaluation were transformed to $\log_{10}$, however, the results are expressed without transformation. Data from the two in vitro experiments were combined, since statistical analysis determined homogeneity of variances according to Barlett's test.

RESULTS

In vitro inhibition of growth and spore production by *P. expansum, C. gloeosporioides* and *M. laxa*

The inhibitory effect of BIOLASTING® was tested at three concentrations (0.5, 1 and 2% v/v) on colony diameter and spore production per plate inoculated with *P. expansum*, *C. gloeosporioides* or *M. laxa* (Table 1). BIOLASTING® at the tree concentrations tested significantly reduced the colony diameter of the three pathogens. The inhibition of in vitro growth caused by BIOLASTING® at 2% v/v varied from 66 to 69% for *P. expansum* and *C. gloeosporioides,* respectively, to 100% inhibition for *M. laxa*. BIOLASTING® at the three doses completely inhibited spore production by *M. laxa* but was not able to reduce it for *P. expansum*, with no significant differences among all the treatments, including the TBDZ (1.2 ppm). BIOLASTING® significantly reduced the spore production per plate of *C. gloeosporioides* but only at the 1 and 2% dosage.

Table 1. Effect of BIOLASTING® applied at three doses on in vitro growth and spore production of *P. expansum*, *C. gloeosporioides* and *M. laxa* after 10 days of incubation.

Treatments	*Penicillium expansum*		*Colletotrichum gloeosporioides*		*Monilinia laxa*	
	CD[1,3]	SP[2,3]	CD[1,3]	SP[2,3]	CD[1,3]	SP[2,3]
Control	56.55 a	1.45×10^9 a	75.67 a	4.14×10^7 a	61.58 a	4.55×10^6 a
BIOLASTING® 0.5%	34.55 b	2.70×10^9 a	54.33 b	1.25×10^7 ab	0.00 b	0.00 b
BIOLASTING® 1%	24.44 bc	2.30×10^9 a	38.00 c	3.53×10^6 bc	0.00 b	0.00 b
BIOLASTING® 2%	19.24 c	2.12×10^9 a	23.33 d	1.58×10^6 c	0.00 b	0.00 b
Thiabendazole 1.2 ppm	32.71 b	2.72×10^9 a	10.08 e	1.75×10^4 d	0.00 b	0.00 b

[1]Growth expressed as colony diameter (CD, mm).
[2]Spore production (SP) per plate.
[3]Mean separation with LSD test ($P<0.05$).

Efficacy tests on fruits under laboratory conditions

BIOLASTING® at any dose tested did not reduce the severity of *P. expansum* infections (Figure 1), but neither did the reference product used (TBDZ). Conversely, BIOLASTING® significantly reduced severity of *C. gloeosporioides* (Table 2) achieving an efficacy between 48 and 78% when applied at 0.5 and 1%, respectively. In these tests, also quality parameters were assessed. The results showed that the product at 1% did not have significant effect on fruit F but led into a significant reduction of the SSC and a significant increase on TA. Similarly, in peaches artificially inoculated with *M. laxa*, BIOLASTING® produced a significant reduction on disease severity at the three doses tested (Figure 2), achieving an efficacy on the severity reduction of 33, 52 and 71% applied at 1, 2 or 3%, respectively.

Table 2. Effect of BIOLASTING® applied at two doses on diseases severity (DS) firmness, soluble solid content (SSC) and titratable acidity on 'Golden' apples inoculated with *C. gloeosporioides*, compared to a reference product based on Thiabendazole 60% (TEXTAR 60T), under laboratory conditions.

Treatments	DS (% rot surface)[1]	Firmness (kg)[1]	SSC (°Brix)[1]	TA (% malic acid)[1]
1 Control	3.36 a	2.42 a	13.58 a	1.94 a
2 BIOLASTING® 0.5%	1.75 b	2.18 bc	12.04 b	2.08 a
3 BIOLASTING® 1%	0.74 b	2.39 ab	12.13 b	2.41 b
4 TEXTAR 60T 0.2%	1.74 c	2.07 c	13.06 a	1.88

[1]Mean separation with LSD test (P<0.05).

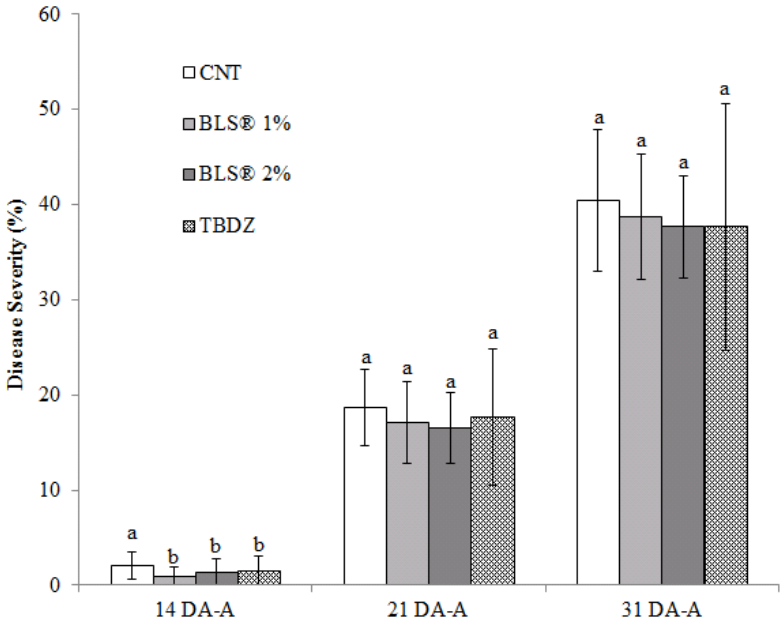

Figure 1. Effect of BIOLASTING® (BLS) at different doses on disease severity of *Penicillium expansum* on 'Golden Delicious' apples stored at 20±2°C during 31 days, compared to a control (CNT) and a reference product based on Thiabendazole (TBDZ) 60% w/v (TEXTAR 60T) (P<0.05), under laboratory conditions.

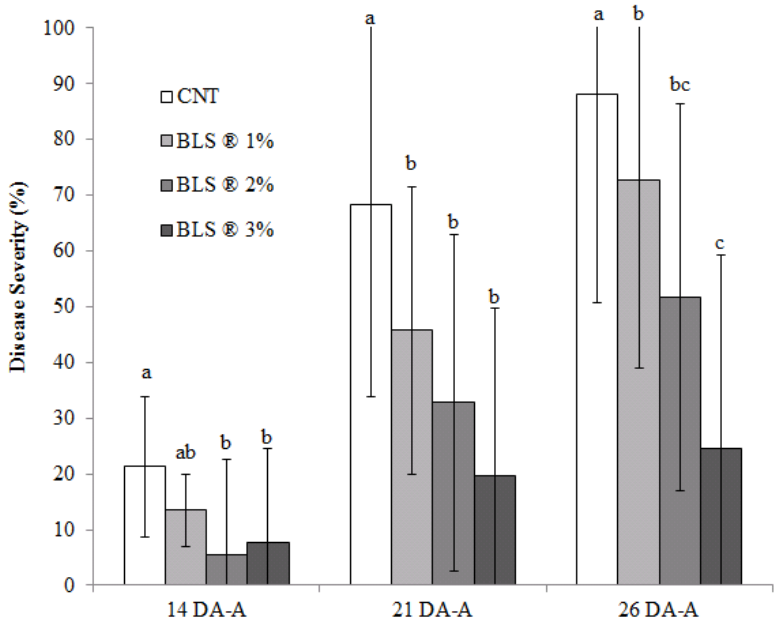

Figure 2. Effect of BIOLASTING® (BLS) at three different doses on disease severity of *Monilinia laxa* on 'Amarillo Tardío' stored at 4±2°C during 26 days compared to a control (CNT) (P<0.05), under laboratory conditions.

Efficacy at semi-commercial trial under cold storage conditions against *Monilinia laxa* on peaches

After 30 days of storage at 0-0.5°C, BIOLASTING® significantly reduced incidence of brown rot in peaches at the two doses and two types of application (drench or spray) used. The best results were achieved by BIOLASTING® applied as a drench at 1.5% v/v (Table 3), reaching 93% of efficacy in reducing incidence on decayed fruit, which was not significantly different from the reference product at the commercial dose. In terms of severity, reduction, all four BIOLASTING® treatments, reached over 90% of efficacy (Figure 3), without significant differences among them and the chemical control.

Table 3. Effect of BIOLASTING® applied as either a drench (D) or spray (S) at two different doses on disease incidence and severity on 'Amarillo Tardío' peaches inoculated with *M. laxa* compared to a reference product based on Fluidoxinil 23% under semi-commercial conditions.

Treatments	Incidence (%)[1]	Severity (%)[2]
1 Control	72.00 a	26.00 a
2 BIOLASTING® 1% D	18.30 b	1.50 b
3 BIOLASTING® 1.5% D	4.80 cd	0.30 b
4 BIOLASTING® 1% S	18.80 b	1.70 b
5 BIOLASTING® 1.5% S	9.30 c	0.90 b
6 SCHOLAR 0.25% D	1.00 d	0.10

[1]Mean separation with LSD test (P<0.05).
[2]Disease severity expressed as percentage of rot surface.

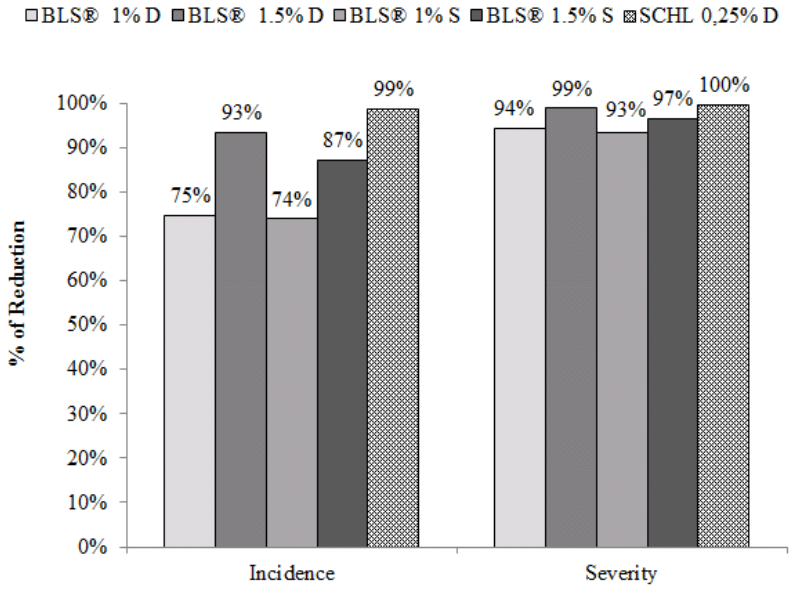

Figure 3. Effect of BIOLASTING® (BLS) at two doses (1 and 1.5%) applied with two different methods (D, drench; S, spray) on the reduction of brown rot incidence and severity on artificially inoculated peaches at semi-commercial conditions after 30 days of storage at 0-0.5°C.

DISCUSSION

BIOLASTING® is a complex formulation, whose efficacy is not due to a single compound. In in vitro assays BIOLASTING® applied at a 2% dosage reduced colony diameter of *P. expansum* by 65% but did not reduced blue mould severity when it was assessed in vivo. Discrepancies between in vitro and in vivo tests in other systems have been frequently reported. In the *P. expansum* tests on 'Golden Delicious' apples, the final severity reached was above the 40% of fruit surface, caused by an inoculation with a high pathogen concentration (10^6 spores mL^{-1}). Additional work should be done with lower doses of the pathogen under controlled storage conditions.

The severity reached in the *C. gloeosporioides* tests on 'Golden Delicious' apples was quite low, between 3 and 3.5% of fruit surface, nevertheless, BIOLASTING® applied at 2%, reduced severity by more than a 50%. In these trials, fruit chemical quality parameters were also assessed. BIOLASTING® changed the SSC and TA when applied at 2%. In particular, SSC was lower than in control or TBDZ treated fruit either with BIOLASTING® at 1 or 2%. Whereas, TA on fruit treated with BIOLASTING® at the higher tested dose significantly increased, compared to the control and the reference product treatment. The changes in these two parameters (SST and TA) led to a better state of preservation and a high quality of the fruit treated with BIOLASTING® at 2%.

The effectiveness of BIOLASTING® against *M. laxa* on peaches at laboratory tests was lower than at semi-commercial trial. The product applied at 1% dosage only get a 33% of reduction in terms of diseases severity, while in semi-commercial trials the same dose reached an effectiveness of 94%. This could be due to the storage conditions, where lower temperatures were maintained (of about 0-0.5°C), compared to the storage conditions of the laboratory trial (4°C). The disease severity on the semi-commercial trial was severe because all fruits were artificially infected with a suspension of *M. laxa* at a relatively high concentration (10^4 spores mL^{-1}). However, the incidence of decayed fruits was reduced in all BIOLASTING® treatments in more than 70%, reaching a 93% of effectiveness in BIOLASTING® applied as drench at 1.5%. In addition, disease severity was reduced over a

90% by all BIOLASTING® treatments.

TBDZ is one of the most common fungicides used in postharvest control of fungal rots. The effectiveness of BIOLASTING® was higher to that of TBDZ at commercial doses against *C. gloesosporioides* on 'Golden Delicious' apples. In semi-commercial trials, BIOLASTING® reached a comparable effectiveness in reducing brown rot incidence and severity on peaches to that of Fludioxinil (exceptionally authorized in Spain for stone fruit postharvest disease control), indicating that this product could be used as a substitute or at least a complement to chemicals against *M. laxa* on peaches and probably *C. gloeosporioides* on apples. Further studies must be performed to confirm the results in this latter pathosystem.

Literature cited

Neri, F., Mari, M., Brigati, S., and Bertolini, P. (2007). Fungicidal activity of plant volatile compounds for controlling *Monilinia laxa* in stone fruit. Plant Dis. *91* (*1*), 30–35 http://dx.doi.org/10.1094/PD-91-0030.

Ortuño, A., Báidez, A., Gómez, P., Arcas, M.C., Porras, I., García-Lidón, A., and Río, J.A.D. (2006). *Citrus paradise* and *Citrus sinensis* flavonoids: their influence in the defence mechanism against *Penicillium digitatum.* Food Chem. *98* (*2*), 351–358 http://dx.doi.org/10.1016/j.foodchem.2005.06.017.

Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H., and Vuorela, P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int. J. Food Microbiol. *56* (*1*), 3–12. PubMed http://dx.doi.org/10.1016/S0168-1605(00)00218-X

Santas, J., Almajano, M.P., and Carbo, R. (2010). Antimicrobial and antioxidant activity of crude oniom (*Allium cepa* L.) extracts. Int. J. Food Sci. Technol. *45* (*2*), 403–409 http://dx.doi.org/10.1111/j.1365-2621.2009.02169.x.

Sanzani, S.M., De Girolamo, A., Schena, L., Solfrizzo, M., Ippolito, A., and Visconti, A. (2009). Control of *Penicillium expansum* and patulin accumulation on apples by quercetin and umbelliferone. Eur. Food Res. Technol. *228* (*3*), 381–389 http://dx.doi.org/10.1007/s00217-008-0944-5.

Schena, L., Nigro, F., and Ippolito, A. (2008). Natural antimicrobials to improve storage and shelf-life of fresh fruit and vegetables and cut flowers. In Microbial Biotechnology in Horticulture, Vol. 2, R.C. Ray, and O.P. Ward, eds. (Enfield, NH, USA: Science Publisher), p.259–303.