Kiwifruit Transgenics for *Osmotin* Gene and Inoculation Tests with *Botrytis cinerea* and *Cadophora luteo-Olivacea*

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Keywords: *Actinidia deliciosa*, fungi, latent infection, inoculation test, shelf life

Abstract

***Botrytis cinerea* is a cosmopolitan ascomycete causing gray mould of fruit in numerous crops including kiwifruit *Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson, which shows a relatively high susceptibility to this pathogen. Recently, it has been observed that *Cadophora luteo-olivacea* also causes a post-harvest problem: skin pitting of kiwifruit. Osmotin is a pathogenesis-related (PR) protein, active against phytopathogenic fungi. The present research compared the resistance to both fungi of kiwifruit harvested from 10-years-old 'Hayward' transgenic plants for tobacco osmotin gene with that fruit from wildtype 'Hayward' plants. A first experiment was carried out to undertake pomological and qualitative evaluation of fruits and estimation of the grade of natural resistance to post-harvest damage after twelve months of storage. A second experiment was carried out to evaluate the resistance of stored fruits after separate artificial inoculation with *B. cinerea* and *C. luteo-olivacea*. A divergent degree of resistance to fungi was detected among the transgenic clones and between them and the wildtype plants. In particular the transgenic clones 120 and 122 fruits retained higher quality in shelf-life than those harvested from control plants, demonstrating a good resistance to post-harvest damage after a long period of storage. Concerning the artificial inoculations, the fruits from transgenic clones 171 and 182 showed higher resistance to *B. cinerea* than fruits harvested from wildtype plants, whereas transgenic clones 66 and 155 showed higher resistance to *C. luteo-olivacea*.**

INTRODUCTION

Botrytis cinerea Pers. is a cosmopolitan ascomycete causing floral and vegetative bud rot and gray mould of fruit in numerous crops including kiwifruit, *Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson, which is relatively highly susceptible to this pathogen (Bisiach et al., 1984). It has also been observed that *Cadophora luteo-olivacea* causes skin pitting in stored kiwifruit (Marchi et al., 1994). The factors that promote the spread and growth of both phytopathogenic fungi include high humidity, excessive nitrogen fertilization and the system of plant training, such as the "tendone" (pergola). Osmotin is a pathogenesis-related (PR) protein active against phytopathogenic fungi; the *osmotin* gene, which is usually expressed by all plants under biotic and abiotic stress conditions (Liu et al., 1994; Zhu et al., 1996), has recently been introduced into *A. deliciosa* 'Hayward' (Rugini et al., 1996). This gene was integrated into the genome of *A. deliciosa* by the plasmid vector pKYLX71 carried by *Agrobacterium tumefaciens* strain LBA4404 in leaf discs and petioles of in vitro-grown shoots. A discrete number of transgenic plant clones was obtained, and these were micropropagated and planted in the field for extra vitro evaluation in a standard training system (Rugini et al., 1999, Gutiérrez-Pesce and Rugini, 2008). The aim of this research was to test the resistance to *B. cinerea* and *C. luteo-olivacea* fungi of post-harvested stored kiwifruit from transgenic clones and wildtype 'Hayward'.

MATERIALS AND METHODS

Two different trials were carried out during the seasons 2008 and 2009, on fruits from 'Hayward' transgenic plants over-producing osmotin. The plants are grown in the authorized field of the Experimental Farm, Department of Crop Production, Tuscia University (latitude 42°25' N, longitude 12°08' E, altitude 310 m a.s.l.). In the first trial, kiwifruit from 11 transgenic clones (66, 109, 120, 121, 122, 132, 141, 155, 169, 171 and 182) and the wildtype, all grown in the same orchard block, were evaluated for fruit weight, total soluble solids and titratable acidity and for relative resistance to pathogens after 12 months storage at 4°C. Twenty fruits per clone were used in this experiment.

In the second trial carried out in 2009, five transgenic clones and wildtype 'Hayward' were compared for resistance of fruit, after 5 months storage at 4°C after separate inoculation with *B. cinerea* and *C. luteo-olivacea*. Four transgenic clones, 66, 155, 171 and 182, were chosen for their high natural resistance to the long period of storage, while clone 121 were chosen as a "minus" variant clone with low natural resistance, as observed in the first trial. The clones 120 and 122 were excluded from the second trial because they carried insufficient fruits in 2009. Four droplets of *B. cinerea* or *C. luteo-olivacea* spore suspensions (50 µl with $5x10^5$ spores ml^{-1}) were placed on the dorsal position of wounded kiwifruit which were then placed in plastic boxes and incubated under controlled conditions (R.H. 90%, temperature 20°C). At 14 days after inoculation the external diameter of lesions was measured and at 21 days the diameter of lesions and the weight of soft rot tissues were recorded and the Disease Index (DI) calculated. DI was based on a 15 point scoring scale considering as parameters the diameter of lesions, the tissue texture and the presence/absence of mycelium or depressions on the fruit surface. Six fruits per each genotype and each artificial inoculation were used and two replicates per test were performed.

Statistical Analysis

The pomological and qualitative data collected in the first trial were analysed by means of the analysis of variance, using the procedure SYSTAT MGLH (Wilkinson, 1998), considering genotype as the variable. Least significant difference (l.s.d. $P = 0.05$) for the comparison of the means was then calculated.

Data collected in the second experiment were expressed as a mean value of two replicates and the standard error was also calculated.

RESULTS AND DISCUSSION

Table 1 shows the physical condition and the incidence of external mould of fruits stored for 12 months at 4°C during the first trial, starting in November 2008. Fruits of clones 120 and 122 were totally firm (Fig. 1), followed by fruits of clones 182, 171 and 155 that showed a high incidence of fruits totally firm and only a low percentage of soft fruits (5.6, 10.5, and 21.1%, respectively). Likewise, clone 66 had a high incidence of firm fruits (77.8%) but also fruits that were soft, partially soft rotten and totally soft rotten, though in low numbers (Table 1). Clones 141 and 121 showed medium natural resistance to damage during the long period of storage, while 169 and 132 showed a high incidence of damaged fruits. Only in clone 109 were all fruits showing a complete tissue maceration at the end of the trial with a performance definitely worse than that of the control (wildtype 'Hayward'). Clones 66, 120, 122, 155 and 182 did not show external mould differently to clones 109 and 32 and the control that showed the highest incidence of external mould on stored fruits (Table 1). The other clones were characterized by a variable amount of external mould. There were differences between clones in fruit fresh weight (FW) and soluble soluble solids content of the juice (TSS), but not in pH and titratable acidity (TA) (Table 2). The TSS ranged from values of 16.80 °Brix in 109 to 12.02 °Brix in control, while the TSS/TA ratio ranged between 10.15 of 155 to 7.31 of control (Table 2).

In the second experiment, the reactions of five transgenic enriched *osmotin* clones to the inoculated infections of *B. cinerea* and *C. luteo-olivacea* were expressed as disease

index (DI) (Fig. 2). The highest value of DI for each fungi, as tested 21 days after artificial inoculations, were found in clone 121. Otherwise, clones 171 and 182 showed the lowest DI to respect at *B. cinerea*, while clones 66 and 155 were characterized by the lowest values of DI for *C. luteo-olivacea* (Fig. 2). Focusing on the results obtained after the artificial inoculation with *B. cinerea* only (Fig. 3), transgenic clones 171 and 182 (Fig. 5) showed higher resistance than 'Hayward' wildtype, considering both diameter of the lesions and soft rot tissue incidence. Also clone 155 showed high resistance but only in terms of soft rot tissue incidence. As shown in Figure 4, clones 155 and 66 were characterized by the highest resistance to *C. luteo-olivacea,* mainly as measured by the lowest diameter of the lesions presents in the fruit 21 days of inoculation. Fruits of lclone 155 were also characterized by the lowest incidence of soft rot tissues (Fig. 6). The highest susceptibility to this phytopathogenic fungi was observed in clone 121 with an incidence of soft rot tissues of more than 50%, 21 days of inoculation (Fig. 4).

CONCLUSIONS

There are no comprehensive reports on the existence and/or anti-microbial effects of osmotin (or other thaumatin-like [TL] proteins) in kiwifruit. The function of osmotin within this crop is unknown and more research work is therefore required to determine how this protein can plays a role in resistance of kiwifruit to fungi, as well as probably in taste. In the clones showing acquired resistance it could be assumed that the *osmotin* gene is more expressed and that the concentration and/or turnover of the protein produces changes in the fungal cell wall organization (Yun et al., 1997, 1998). Detailed work is required to unravel the behaviour of clones 66, 155, 171 and 182, that appear to be more resistant to the post-harvest damage caused by the two phytophatogenic fungi studied. Analogous investigations should be carried out to elucidate the behaviour of clones 120 and 122, that even though they were not analysed in the second trial have shown the highest resistance to the damage generated from long periods of storage.

ACKNOWLEDGMENTS

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Tables

Table 1. Physical condition (A = totally firm; B = soft; C = partially soft rotten; D = totally soft rotten; E = tissue maceration) and incidence of external mould on fruits of transgenic *A. deliciosa* 'Hayward' clones over-producing osmotin and wildtype (control) stored for 12 months at 4°C.

Genotype	External fruit status (%)					External mould (%)	
	A	B	C	D	E	Absent	Present
66	77.8	5.6	5.6	11.0	-	100.0	-
109	-	-	-	-	100.0	-	100.0
120	100.0	-	-	-	-	100.0	-
121	37.5	31.2	25.0	-	6.2	18.7	81.2
122	100.0	-	-	-	-	100.0	-
132	-	-	20.0	40.0	40.0	-	100.0
141	43.7	43.7	-	12.5	-	93.7	6.2
155	78.9	21.1	-	-	-	100.0	-
169	15.8	52.7	10.5	10.5	10.5	21.0	79.0
171	89.5	10.5	-	-	-	89.5	10.5
182	94.5	5.5	-	-	-	100.0	-
Control	-	-	21.8	31.3	46.9	-	100.0

Table 2. Fresh weight (FW), soluble solids content (TSS), titratable acidity (TA) and TTS/TA ratio determined in the juice of fruit of transgenic *A. deliciosa* 'Hayward' clones over-producing osmotin and wildtype (control) stored for 12 months at 4°C. Values are expressed as a mean of replicates.

Genotype	FW (g)	TSS (°Brix)	pH	TA (g malic acid/100 g fw)	TSS TA^{-1}
66	104.05	14.30	3.37	1.62	8.87
109	118.38	16.80	3.42	1.74	9.66
120	96.02	14.45	3.45	1.58	9.14
121	94.42	13.95	3.49	1.72	8.14
122	94.00	13.00	3.47	1.75	7.45
132	74.51	14.15	3.32	1.80	7.86
141	86.19	13.95	3.43	1.48	9.54
155	98.38	15.85	3.40	1.57	10.15
169	99.70	13.35	3.59	1.57	8.53
171	80.40	13.85	3.34	1.54	9.07
182	102.14	14.10	3.53	1.52	9.28
Control	91.95	12.02	3.49	1.65	7.31
l.s.d. (p = 0.05)	16.15	1.97	n.s.	n.s.	n.s.

Figures

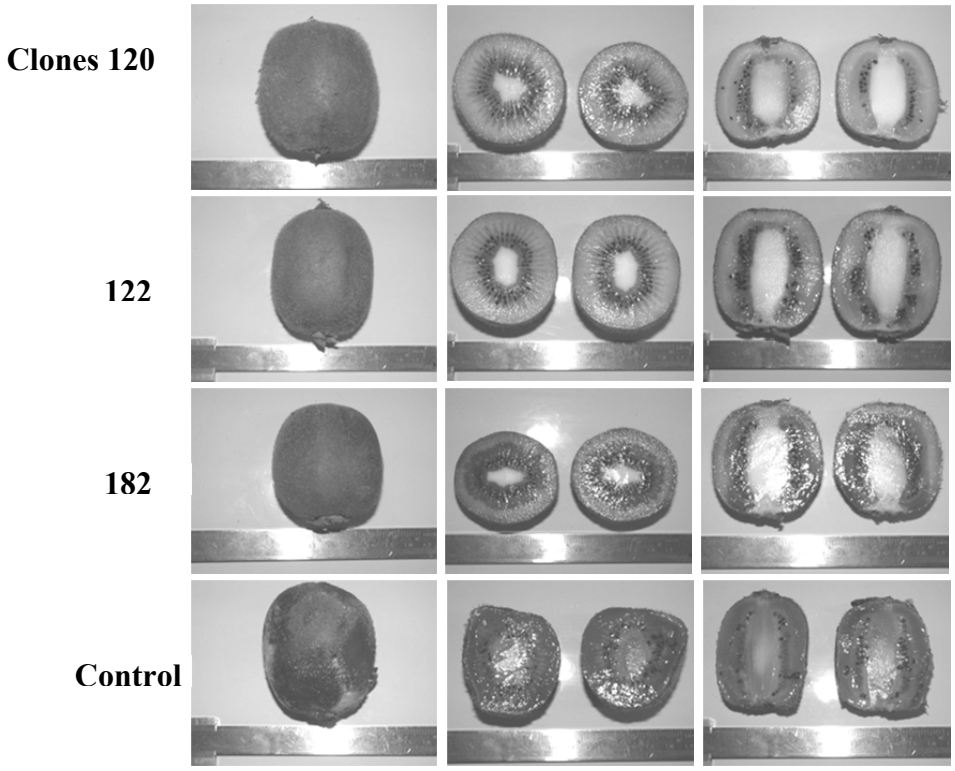

Fig. 1. External and internal appearance fruit of transgenic *A. deliciosa* 'Hayward' clones over-producing osmotin and wildtype (control) stored for 12 months at 4°C.

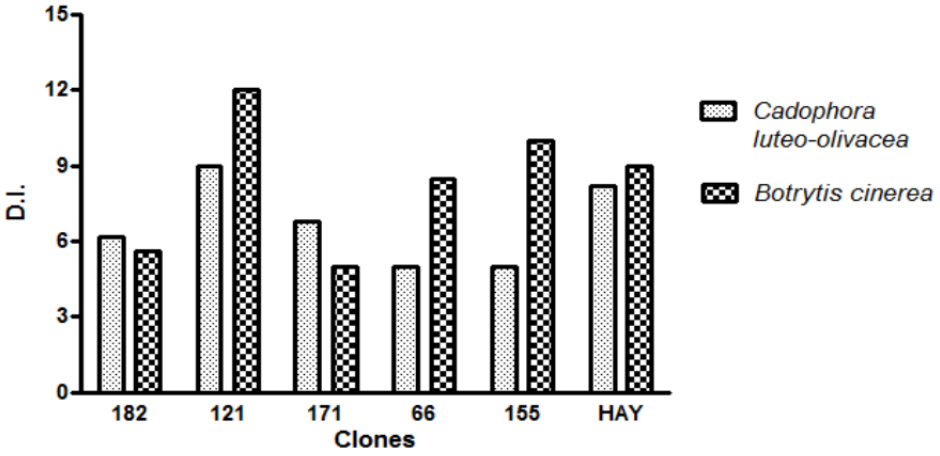

Fig. 2. Disease index (DI) at 21 days after artificial inoculation with *Botrytis cinerea* and *Cadophora luteo-olivacea* of transgenic osmotin over-producing clones of *Actinidia deliciosa* and 'Hayward' wildtype (Hay). DI was based on a 15 point scoring scale considering as parameters the diameter of lesions, the tissue texture and the presence/absence of mycelium or depressions on the fruit surface.

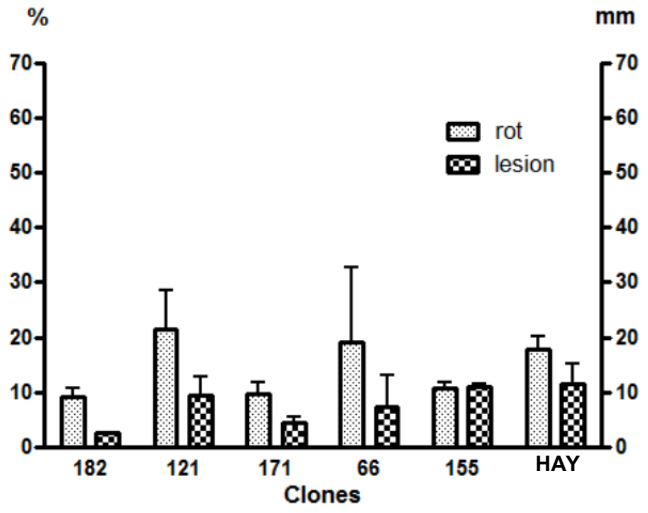

Fig. 3. Evaluation of resistance to *Botrytis cinerea* in fruit of transgenic osmotin over-producing clones of *Actinidia deliciosa* and 'Hayward' (Hay). The evaluation parameters considered were the soft rot tissue incidence (%) and the diameter of the lesion (mm) 21 days after artificial inoculation.

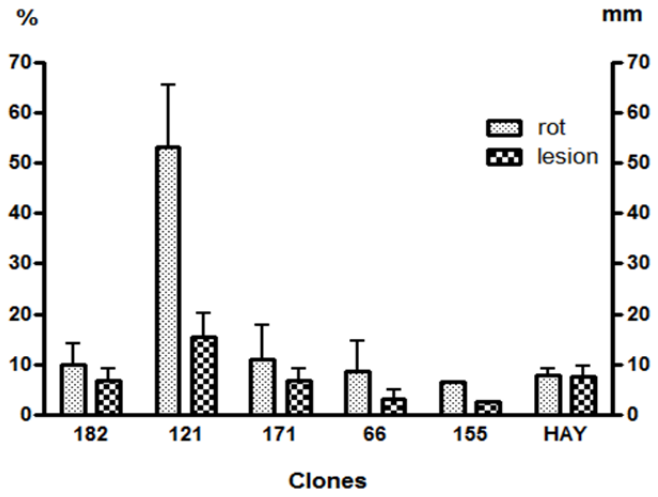

Fig. 4. Evaluation of resistance to *Cadophora luteo-olivacea* in in fruit of transgenic osmotin over-producing clones of *Actinidia deliciosa* and 'Hayward' wildtype (Hay). The evaluation parameters considered were the soft rot tissue incidence (%) and the diameter of the lesion (mm) 21 days after artificial inoculation.

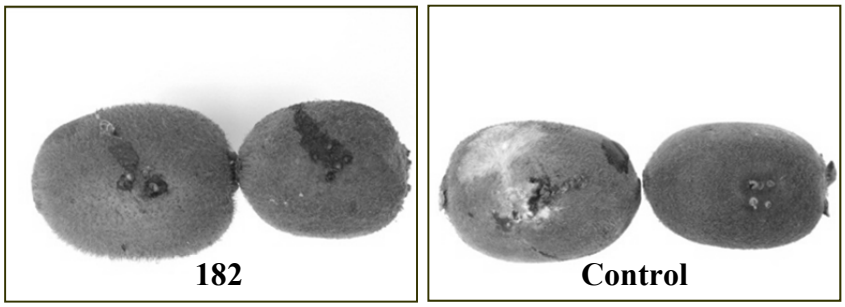

Fig. 5. Kiwifruit clone 182 (left) and 'Hayward' control (right) 21 days after artificial inoculation with *Botrytis cinerea*.

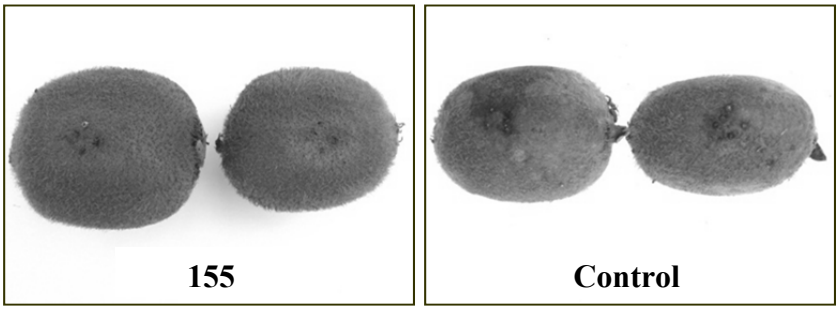

Fig. 6. Kiwifruit clone 155 (left) and 'Hayward' control (right) 21 days after artificial inoculation with *Cadophora luteo-olivacea*.