Combination of ultraviolet-C irradiation and biocontrol treatments to control decay caused by *Penicillium digitatum* in 'Washington navel' orange fruit

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Abstract

The combination of the yeast *Candida oleophila* strain '13L' with ultraviolet-C irradiation evidenced a synergistic effects in reducing *P. digitatum* mould and only 11% of the artificially inoculated wounds were infected. Adversely, when the bacteria *Bacillus subtilis* strain 'B160' was combined with ultraviolet-C irradiation no synergistic effect was achieved. By using only yeast, bacteria or ultraviolet-C treatments the decay percentage was reduced by 79.6 and 75%, respectively. The phytoalexin scoparone accumulation was high in all treatments where UV-C was applied but the highest values were found when combined with the yeast. Population growth of bacteria in vivo was halved when fruit was irradiated, whereas direct irradiation of bacteria did not affect their growth in vitro. An inhibitory effect of the phytoalexin toward the bacteria is suggested as the reason for the growth inhibition in vivo when the bacterial treatment was combined with UV-C irradiation.

INTRODUCTION

Chemical control of pests has dominated the scene, but its overuse has adverse effects on human health and environment, as well as on international trade (Fragenberg, 2000). The interest in alternative approaches such as biological control, use of compounds generally recognized as safe (GRAS) and physical means such as UV irradiation is accordingly great. Antagonistic microorganisms play an important role in this contest, especially in the control of postharvest moulds (Wilson et al., 1993). Several works have reported promising results on a large number of crops with various microorganisms such as yeast (Lima et al., 1995), bacteria (Bull et al., 1997) and fungi (Borrás and Aguilar, 1990). Four commercial formulations [Aspire (Ecogen INC., Langhorn, PA); YieldPlus (Anchor Yeast, Cape Town, South Africa); BIOSAVE-110 and BIOSAVE-111 (EcoScience, Orlando, FL)] are already available and used to prevent pre and postharvest diseases. Nevertheless, as for all living organisms environmental conditions are crucial and low temperatures or modified atmosphere conditions applied after harvest may act adversely on the efficacy of these biocontrol agents. . So, many antagonists that appear promising under laboratory conditions may show their limitations under a wider range of environmental conditions such as in commercial or semi-

commercial type experiments. Often, this could also be due to their lack of curative activity, as their efficacy is much reduced or nil when antagonists arrive on wounds after pathogens Janisiewicz and Korsten, 2002).

Accordingly, there is a great need for a search of combined treatments to strengthen the activity of the antagonists. Physical means are less environment-dependent and, among these, heat treatment and ultraviolet-C light (UV-C - 254 nm) applied to citrus fruit reduced rot magnitude during storage (Schirra et al., 2000; D'hallewin et al., 2000). In citrus fruit, UV-C light was reported to affect the postharvest pathogens by enhancing natural resistance (Ben Yehoshua et al., 1992; Kim et al., 1991; Rodov et al., 1992). The application of antagonistic yeast before fruit curing (37°C for 24 h) improved the control of *Penicillium* spp. during storage of grapefruit (D'hallewin et al., 1999a). The beneficial effect was due to a faster growth and colonization of the lesions at fruit surface level. UV-C light is easily applied to fresh commodities but depending upon harvest time the hormetic dose may be close to the deleterious one (D'hallewin et al., 1999b). Currently, single alternative treatments did not reach comparable control to chemicals on postharvest moulds, thus the struggle to keep under control the pathogens is still open.

The present research was performed in order to assess the feasibility to combine UV-C treatments with biocontrol agents such as yeast and bacteria to control green mould decay during storage of orange fruit.

MATERIALS AND METHODS

Fruit

The investigation was carried out on 'Washington navel' orange [*Citrus sinensis* (L.) Obsek] fruit harvested in mid-January in an organically cultivated grove (South-western Sardinia, Italy, 39° 55' N). Fruit were transported the same day to the laboratory and six-hundred unblemished oranges were randomly chosen, surface disinfected for 3 min in a diluted bleach solution of sodium hypochlorite (2% active chlorine), rinsed with deionized (DI) water and left to dry at room temperature overnight.

Artificial inoculation

Wounding of all fruit was achieved 24 h after harvest by injuring the flavedo and albedo at four equatorial points with a steel rod (3x3 mm) and inoculation was performed 1 h later. To inoculate the fruit a *Penicillium digitatum* wild type isolate was grown in the dark at 25°C on potato dextrose agar (PDA). Conidial suspension was obtained from a 7-day-old culture submersed with a DI water-Tween 80 (0.05% w/v) solution. Conidial concentration was adjusted to $2x10^6$ spores ml^{-1} by a hemacytometer and 20 µl of the suspension was injected to each wound.

Antagonist preparation

Candida oleophila isolate '13L' (Arras et al., 1998) and *Bacillus subtilis* isolate 'B160' (Arras, 1993) grown in culture tubes slants were transferred to a 1000-ml flask containing 600 ml of nutrient yeast extract dextrose broth (NYDB) prepared as directed by the manufacturer and incubated on a shaker (150 rpm) at 25°C for 48 h. The growth media was removed by centrifugation (9.000 x *g* for 10 min) and the cell pellet was resuspended in DI

water, vortexed and centrifuged twice. The cell pellet obtained was finally suspended in a sterile physiological solution (0.9% NaCl) and the concentration adjusted to $1x10^7$ colony-forming units (cfu) ml^{-1} by a hemacytometer.

UV-C treatment

UV-C light 254 nm was applied to the fruit by a device having 4 light bulbs of 3.6 W (G15T8, Tana Ind., Tel Aviv. IL) each, and the fruit was placed at a distance of 20 cm from the light sources. No increase of temperature was monitored during the treatment and the chamber was ventilated in order to avoid ozone accumulation. According to previous results aimed to establish the most effective dose avoiding treatment damage (D'hallewin et al., 1999b), the dose applied was of 1.5 KJm^{-2}.

Experimental plan

Six hundred wounded and inoculated fruits were kept at 25°C and 95% relative humidity (RH) for 12 h, then divided into 6 groups of 100 fruit. The first two groups were treated with the yeast (isolate '13L') by injecting into each wound 20 µl of a $1x10^7$ cells/ml of the yeast. The third and fourth group were treated with the bacteria (isolate 'B160') in the same way as with yeast. The fifth group was treated with UV-C, and the sixth group did not receive any treatment (control). Four h after antagonist application the fruit of the second and fourth group were treated with UV-C light. Following treatments all fruit was kept up to 24 h at 25°C then, placed in one-layer boxes and stored in dark, ventilated rooms at 8°C and 95% RH with one complete air change each hour.

Population dynamic in vivo

To perform *in vivo* growth dynamic studies of the yeast and bacteria isolates three replicates of 5 fruit for each treatment were wounded in 4 points at the equatorial area, inoculated, UV-C treated and stored as described before. Population growth (cfu/wound) was monitored after 1, 2, 3, and 5 days by removing a cylinder (6 x 5 mm) of tissue (flavedo + albedo) around the wound by means of a cork borer and a small spatula. At each assessment 3 replicates of 5 samples were homogenized in 10 ml of a sterile NaCl (0.9%) solution. A sample of the suspension was taken and an appropriate serial dilution was spiral-plated on PDA. Plates were incubated for 2 days at 25°C and the number of colonies was counted.

Population dynamic in vitro

The population dynamic (cfu/ml) of the yeast '13L' and bacteria 'B160' was performed in vitro at 8°C on nutrient broth (NB) (Acumedia, laboratories Inc. Baltimore, MY, U.S.A.) inoculated either with irradiated or un-irradiated antagonists. Cfu/ml counts were performed as for the in vivo dynamic study.

Decay monitoring

The infection magnitude was checked after eight days and expressed as percentage of infected wounds.

Phytoalexin detection and quantization

The amount of the phytoalexin scoparone (6,7-dimethoxycoumarin) at the wound site (flavedo+albedo) was determined immediately after irradiation,

after 12 h and at 1, 2, 3 and 7 days post-treatment by HPLC quantitative analysis according to D'hallewin et al. (1999a).

Statistical analysis

Percentage of decayed wounds were transformed to Bliss angular values and subjected to variance analysis using MSTAT-C software (Michigan State University, E. Lansing, Mi, 1991). When needed, separation of means was performed by Duncan's test at $P \leq 0.05$.

RESULTS

Population dynamic in vitro and in vivo

The growth dynamic of bacteria evidenced differences between the in vitro and in vivo experiments (Fig. 1). A clear growth of both antagonists was found in vitro, the yeast having a significantly greater increase compared to bacteria (Fig. 1). The irradiation of either antagonists with 1.5 kJm^{-2} did not affect the growth rate and differences between irradiation and no-irradiation were negligible. A small effect was noted when bacteria growth was checked 12 h after irradiation while after 7 days the difference was not significant. Considering the growth rate of the yeast within wounds it was noted that also under these conditions the population dynamic was not affected by irradiation and values were not different from the ones achieved in vitro (Fig. 1). Bacteria had a completely different behaviour showing a significant increase of cells within the first 24 h followed by a rapid decrease that was significantly greater when fruit was irradiated.

Effect on decay

The inoculation experiment evidenced a small reduction of the green mould by all single treatments compared to un-treated inoculated fruit. The percentage of infected wounds was reduced by 79, 55 and 75% using yeast, bacteria or UV-C irradiation, respectively (Table 1). The combination of yeast followed by UV-C irradiation reduced infection markedly in a statistically significant and synergistic effects compared to any of the single treatments. Only 11% of the inoculated wounds were actively infected. No synergistic effect was observed when UV-C treatment was performed after bacteria application and the infection magnitude was not different from that of the single UV-C treatments (Table 1).

Phytoalexin detection and quantization

The accumulation of scoparone in wounded tissue inoculated with *P. digitatum* reached 65μg g^{-1} fresh weight (FW) 5 days post-irradiation (Fig. 2). A similar induction was found for inoculated wounds treated with bacteria, whereas when combined with UV-C irradiation the accumulation was nearly four times as much (210 μg g^{-1} FW). When UV-C irradiation was applied alone the amount of scoparone detected 7 days after treatment was slightly, but significantly higher, compared to bacteria followed by UV-C (Fig. 2). The bacteria did not increase the level of scoparone neither alone nor combined with UV-C treatment. On the contrary, the yeast alone was able to induce a considerable amount of scoparone, nearly twice the amount detected in control fruit. When this antagonist was combined with UV-C the accumulation

increased synergistically reaching very high levels of scoparone (380 μg g^{-1} FW) 7 days post UV-C treatment.

DISCUSSION AND CONCLUSION

The attempt to improve alternative ecologically friendly treatments by either combining or by the single application of these different approaches evidenced some difficulties in not achieving the expected decay control. Treatment damages were below 3% and most of it was of low severity, being acceptable. A significant benefit was achieved by combining the yeast '13L' with the physical treatment. This combination resulted in an increase of the decay reduction efficacy. Infection percentage by *P. digitatum* was lowered by nearly 80% compared with the control that had 93% of infected wounds. Earlier reports (Rodov et al., 1994) on the mode of action of bio-control antagonists showed the ability of some yeasts, but not of bacteria antagonists to induce phytoalexins in *Citrus* spp. without considerable phytoxicity. This work confirmed that induction of the accumulation of scoparone (Fig. 2) and the limited phytoxicity also apply to the yeast isolate '13L'. It was also demonstrated by its population growth in vivo (Fig. 1). Additionally this bio-control agent acted as a phytoalexin inducer and the accumulation of scoparone was synergistic when yeast was combined with the UV-C treatment.

Adverse results were obtained when UV-C irradiation followed bacteria application. The lack of rise in the bacterial efficacy in reducing decay and the weak in vivo population growth leads to the speculation that environmental factors within the wound affected negatively the bacteria. Similarly the in vitro experiment evidenced also no effect of the irradiation on the colony growth. In addition the scoparone accumulation induced by UV is possibly one of the adverse environmental factors since in vitro experiments carried out by Jurd et al. (1971) showed a bacterio-static effect of this compound.

In conclusion it is evident that results are far from convincing to replace the traditionally used imazalil or thiabendazole with the alternative treatment of combined UV-C and biocontrol antagonist. A significant improvement in decay reduction was achieved by combining the bio-control yeast strain "13L" with UV-C irradiation compared to either single treatment. Furthermore, reasonable decay control is achieved by another combination of UV-C with hot water (Ben Yehoshua et al., 2004). They suggest that their treatment may be the reply to a real problem of decay control of fruits such as kumquat in which the effective chemical fungicides are not allowed. Furthermore possibly for such fruits the addition of the biocontrol antagonists may be helpful.

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Table 1. Percentage decay on 'Washington navel' orange fruit artificially inoculated by *P. digitatum* and treated singularly with the antagonist yeast "13L", the bacterium "B160" and UV-C or in combination[w].

	Treatment					
	none	13L[x]	B160	UV-C	A5/UV-C	B160/UV-C
Infected wounds (%)	93[y] ± 2	21 ± 4	45 ± 5	25 ± 7	11 ± 3	23 ± 5

[w] Inoculation with 20 µl of a $2x10^6$ spores ml^{-1}; infection was monitored after 8 days and fruit was stored at 8°C and 95% RH.

[x] Each value is the mean ± standard error of 4 replicates of 100 wounds each.

[y] "13L" strain of *C. oleophila* and "B160" strain of *B. subtilis* were used at $1x10^7$ colony-forming units (cfu) ml^{-1}, UV-C irradiation was performed at 1.5 kJm^{-2}.

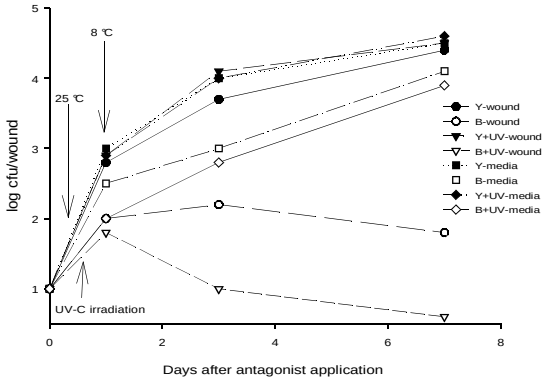

Fig. 1. Population dynamic of the yeast *C. oleophila* (13L) and the bacterium *B. subtilis* (B160) on wounded 'Washington navel' orange fruit or PDA media subjected or not to ultaviolet-C irradiation (1.5 kJm^{-2}) and stored at 8°C and 95% RH .

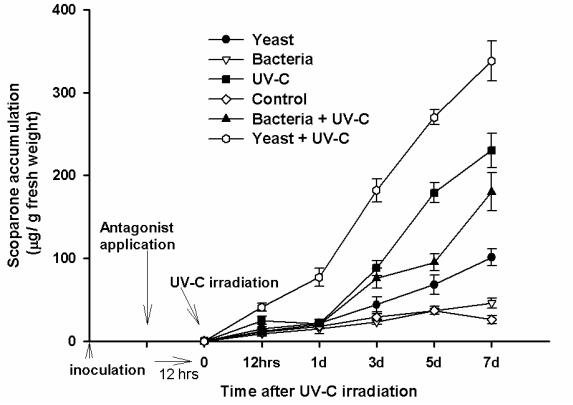

Fig. 2. Scoparone accumulation following single or combined treatments with the yeast *C. oleophila* (13L), the bacterium *B. subtilis* (B160) and ultaviolet-C light (1.5 kJm^{-2}), in 'Washington navel' orange wounded and inoculated with *P. digitatum*, stored at 8°C and 95% RH. Bars indicate the confidential interval (*P*=0.05).