SHORT COMMUNICATION

THE NON-TRADITIONAL GROWTH REGULATOR PECTIMORF IS AN ELICITOR OF DEFENSE RESPONSES AND PROTECTS ARABIDOPSIS AGAINST *BOTRYTIS CINEREA*

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SUMMARY

Pectimorf is a non-traditional growth regulator produced in Cuba by partial enzymatic degradation of the cell wall of citrus fruit rinds. It is mainly composed of oligogalacturonides (OGs) and is currently used for *in vitro* micropropagation of different crops. Since OGs are known to elicit plant defence responses and are able to protect plants against fungal infection, we have investigated whether Pectimorf also has elicitor properties in *Arabidopsis thaliana*. The activity of Pectimorf was similar to that observed with similar doses of OGs in different bioassays (induction of gene expression, elicitation of oxidative burst, activation of MAP kinases). Furthermore, Pectimorf pre-treatments increased resistance of Arabidopsis leaves against infection by *Botrytis cinerea*. Pectimorf, as previously observed with OGs, also showed antagonistic effects with exogenous auxin. Taken together, these data indicate that Pectimorf is a potent elicitor of plant innate immunity and may be used as an inexpensive natural product to protect crops against diseases.

Key words: Pectimorf, oligogalacturonides, elicitors, *Botrytis cinerea*, innate immunity.

Elicitors are chemical signals that activate plant defence responses against pathogen infection. Several well-characterized elicitors are structural microbial components like chitin from fungal cell walls, β-glucans derived from the cell wall of oomycetes, the protein flagellin, the principal component of the bacterial flagella, and the bacterial elongation factor EF-Tu. These molecules are often referred to as microbe-associated molecular patterns (MAMPs) and their perception triggers in plants an innate immune response that leads to the accumulation of phytoalexins, reactive oxygen species (ROS) and pathogenesis-related (PR) proteins (Boller and Felix, 2009). These responses contribute to restrict the pathogens and increase plant resistance to subsequent infections. Not only MAMPs but also endogenous elicitors can be released from the plant cell upon pathogen infection. Several studies indicate that α-(1-4)-D-oligogalacturonides (OGs) released upon degradation of homogalacturonan (Hahn *et al*., 1981), a major component of pectin, trigger defense responses in different plant species, such as accumulation of phytoalexins, glucanase and chitinase (Davis *et al*., 1986; Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988). Treatments with OGs reduce symptoms caused by the necrotrophic fungus *Botrytis cinerea* in grapevine (*Vitis vinifera*) and *Arabidopsis thaliana* leaves (Aziz *et al*., 2004; Ferrari *et al*., 2007), suggesting that this class of elicitors could be used as biological inducers of resistance in the field. The molecular components of the perception and transduction of OGs in Arabidopsis have been extensively studied. OGs are perceived by the wall-associated kinase WAK1 (Brutus *et al*., 2010), and activate a robust oxidative burst, mediated by the NADPH oxidase AtRbohD (Galletti *et al*., 2008). In Arabidopsis OGs also trigger the rapid phosphorylation of the MAP kinases (MAPKs) MPK3 and MPK6 (Galletti *et al*., 2011).

Pectimorf is a non-traditional growth regulator produced in Cuba by partial enzymatic degradation of the cell wall of citrus fruit rinds (Cabrera *et al*., 2003). It is mainly composed of a mixture of OGs and is used as an inexpensive substitute for traditional hormones for *in vitro* micropropagation, because it stimulates growth and differentiation of different crops such as tomato, cassava, sugarcane and guava (Plana *et al*., 2003; Ramirez *et al*., 2003; Nieves *et al*., 2006; Suárez and Hernández, 2008). Pectimorf also appears to have positive effects on growth and yield in soil-grown plants. For instance, yield of tomato plants increased by 40% following the application of this growth regulator (García Sahagún *et al*., 2009).

Since partially or fully purified OGs are elicitors of defence in plants, we speculated that Pectimorf may also be effective in inducing most responses typically activated by OGs, and therefore it could be used as an inexpensive and environmentally safe product for crop

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protection. For these reasons, we compared Pectimorf and partially purified OGs for their ability to induce a range of known defense responses in the model plant Arabidopsis. We first investigated the ability of Pectimorf to induce responses typically triggered by OGs in liquid-grown Arabidopsis seedlings. Arabidopsis Columbia-0 (Col-0) seeds were surface-sterilized and placed in multi-well plates containing liquid half-strength Murashige and Skoog basal medium (Sigma-Aldrich, Italy) (Murashige and Skoog, 1962), pH 5.5, supplemented with 0.5% sucrose (about ten seeds in 1 ml of medium per well). Plates were incubated in a growth chamber at 22°C with a 16/8 h light/dark cycle and a light intensity of 120 µmol m^{-2} s^{-1}, and the medium was refreshed after nine days. The subsequent day, seedlings were treated with water, 100 µg ml^{-1} Pectimorf (prepared by the Department of Plant Physiology and Biochemistry of the National Institute of Agricultural Sciences at Mayabeque, Cuba) or with a preparation of OGs containing fragments with DP = 7-12, obtained as previously reported (Bellincampi *et al.*, 2000), at the same final concentration. Accumulation of hydrogen peroxide in the medium was evaluated after 15 min of treatment, using a xylenol orange-based assay (Jiang *et al.*, 1990). Both OGs and Pectimorf caused accumulation of comparable levels of H_2O_2 in the medium (Fig. 1A).

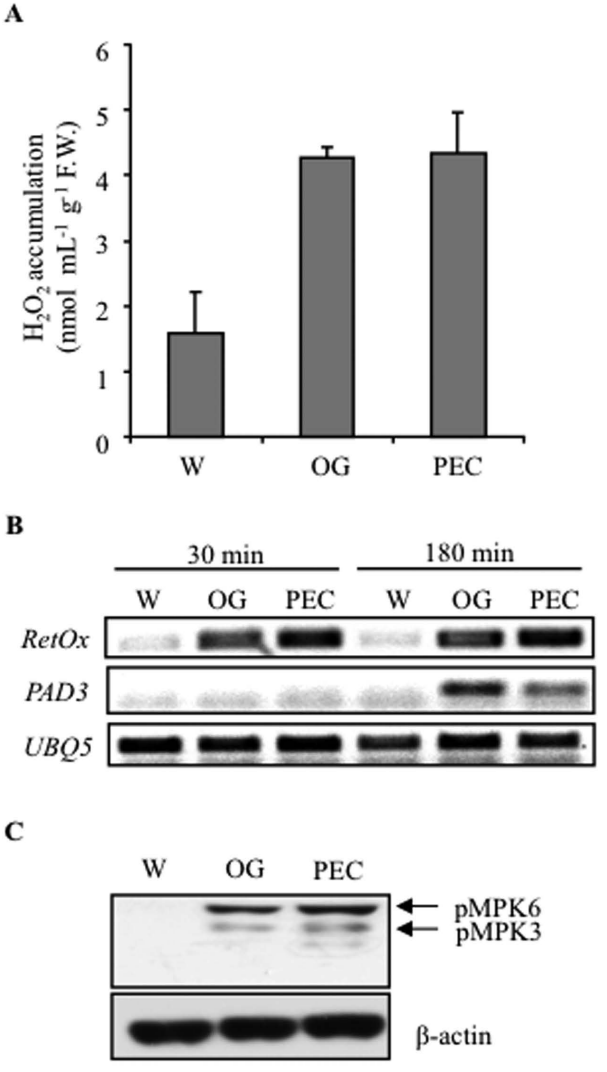

Fig. 1. Pectimorf and oligogalacturonides induce similar defense responses in Arabidopsis. Arabidopsis liquid-grown seedlings were treated with water (W), OGs or Pectimorf (PEC), and activation of defence responses was determined after 15 min (A, C) or at the indicated times (B). (A) Accumulation of extracellular hydrogen peroxide, measured with a xylenol orange-based assay. Results are the mean of three independent experiments made on at least 7 wells containing 10 seedlings each (± SD). (B) Expression of the defence genes *RetOx* and *PAD3*, determined by RT-PCR, using *UBQ5* as reference. (C) Phosphorylation of MPK3 and MPK6, determined by immunoblot analysis with antibodies against phospho-p44/p42 (α-pTEpY), or against β-actin as loading control. Bands corresponding to phosphorylated MAPKs (pMPK3 and pMPK6) are indicated by arrowheads. These experiments were repeated three times with similar results.

Then the expression was evaluated of two genes that are markers of the OG response, namely *RetOx*, that is induced rapidly after elicitor treatment, and *PAD3*, that is typically induced later (Galletti *et al.*, 2008). *PAD3* encodes a cytochrome P450 required for the last step of camalexin biosynthesis, the main Arabidopsis phytoalexin (Zhou *et al.*, 1999), whereas *RetOx* shows homology with enzymes involved in alkaloid biosynthesis and ROS production in other plant species. We treated liquid-grown seedlings with water or with OGs or Pectimorf at the same concentration (150 µg ml^{-1}). Total RNA was extracted from seedlings harvested after 30 and 180 min of treatment, using the Isol-RNA lysis reagent (5 PRIME GmbH, Germany). Expression of the two marker genes and of the housekeeping gene *UBQ5* was determined by semi-quantitative RT-PCR as previously described (Galletti *et al.*, 2008). OGs induced the expression of *RetOx* at 30 min of treatment and the expression was sustained up to 180 min. *PAD3* transcripts significantly increased only at 180 min of treatment. Notably, Pectimorf treatment induced the expression of both marker genes with similar kinetics (Fig. 1B).

Activation of MAP kinases (MAPKs) is another response that is rapidly induced by different elicitors. In particular, OGs trigger a transient phosphorylation of the Arabidopsis MPK3 and MPK6 (Galletti *et al.*, 2011). Therefore the levels were determined of the phosphorylated forms of these two MAPKs in Arabidopsis seedlings treated for 15 min with water, 100 µg ml^{-1} OGs or 100 µg ml^{-1} Pectimorf. Accumulation of the phosphorylated forms of MPK3 and MPK6 was studied by immunoblot analysis using an anti-phospho-p44/p42 antibody that specifically recognizes the phosphorylated forms of these MAPKs (Saijo *et al.*, 2009) as previously described (Galletti *et al.*, 2011). OGs and Pectimorf triggered a similar response, causing a significant accumulation of phosphorylated MPK3 and MPK6 (Fig. 1C).

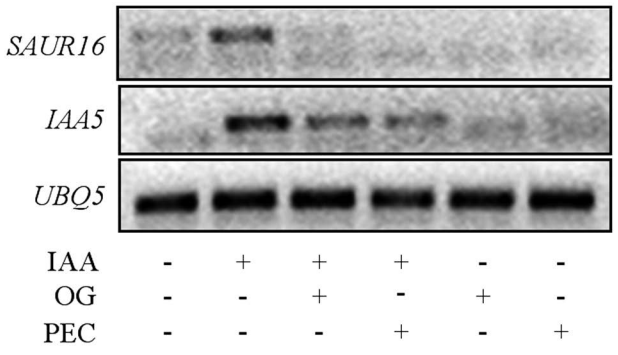

Fig. 2. Pectimorf inhibits the expression of auxin-regulated genes. Expression of the indicated genes was determined by RT-PCR in Arabidopsis seedlings treated for 1 h with water (-) or 1.5 µM IAA ± OGs or Pectimorf (PEC). Results are representative of three independent experiments.

OGs have been extensively characterized also for their effect on plant growth and development, and in particular for their activity as antagonists of auxin. For instance, OGs suppress indole acetic acid (IAA)-induced formation of adventitious roots in leaf explants (Bellincampi *et al*., 1993; Savatin *et al*., 2011), and inhibit IAA-induced expression of early marker genes (Savatin *et al*., 2011). The molecular mechanism of the anti-auxin activity of OGs is not fully elucidated, though the involvement of miRNAs or of the stabilization of AUX/IAA inhibitors in this process has recently ruled out (Savatin *et al*., 2011). Considering that Pectimorf is used as a substitute of plant growth regulators in micropropagation, we investigated whether Pectimorf exerts an anti-auxin effect in Arabidopsis plants. In particular, the expression was monitored of two early auxin-inducible genes, *SAUR16* and *IAA5*, in Arabidopsis liquid-grown seedlings treated with 1.5 µM IAA alone or in presence of 100 µg ml^{-1} OGs or Pectimorf. In the absence of elicitors, expression of both genes was induced after 1 h of treatment with IAA (Fig. 2). In the presence of either OGs or Pectimorf, transcript levels of *SAUR16* and, to a lesser extent, of *IAA5* were significantly reduced, compared to seedlings treated with auxin alone (Fig. 2).

Our results indicate that Pectimorf is able to activate, in Arabidopsis seedlings, responses that are largely overlapping those activated by similar doses of OGs. Therefore we tested whether Pectimorf also protects Arabidopsis against pathogen infection. Soil-grown four-week-old Arabidopsis plants were sprayed with water or with 150 µg ml^{-1} Pectimorf, and, after 24 h, rosette leaves were inoculated with a *B. cinerea* spore suspension as previously described (Ferrari *et al*., 2007). Measurement of the area of the lesions caused after two days by the fungus on the inoculated leaves revealed that Pectimorf treatment significantly reduces diseases symptoms, with an average lesion size of about 50% of the size observed in control-treated plants (Fig. 3).

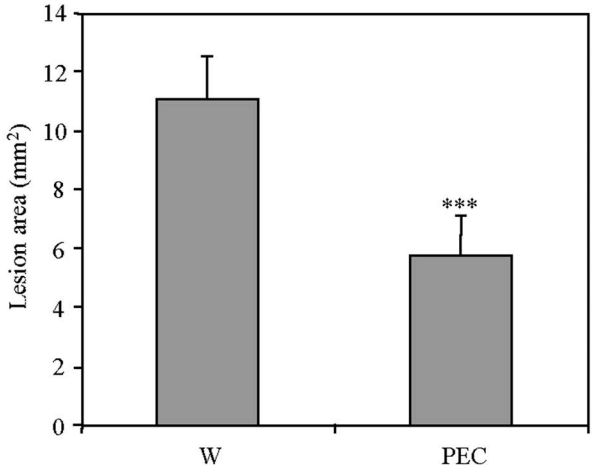

Fig. 3. Pectimorf protects Arabidopsis plants against fungal infection. Arabidopsis plants were treated with water (W) or Pectimorf (PEC) and, after 24 h, were inoculated with *B. cinerea*. Lesion area was measured 48 h after inoculation. Bars indicate average lesion size ± standard error (n ≥ 10). Asterisks indicate statistically significant differences, according to the Student's t-test ($P < 0.01$). This experiment was repeated twice with similar results.

In conclusion, our data indicate that Pectimorf is a potent elicitor of the plant innate immunity and activates responses that are qualitatively and quantitatively comparable to those induced by OGs. Compared to purified OGs, preparation of Pectimorf is less expensive, and may be employed in crop protection as a substitute for currently used pesticides. Field studies on cultivated plants will be conducted in order to assess the effectiveness and the durability of Pectimorf protection.

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