EFFICACY OF PHENOLIC-RICH EXTRACTS FROM LEAVES OF PEPPER LANDRACES AGAINST ALTERNARIA LEAF BLIGHT OF TOMATO

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SUMMARY

Plant-derived antifungals have an interesting potential to be used for the sustainable management of plant diseases. In this work, seven different local genotypes of Capsicum annuum, diffused in agricultural areas of Campania Region (Italy), were explored for their potential in providing phytochemical extracts suitable for antifungal applications. Bioactive hydro-methanolic extracts has been assessed for phenolic composition and antioxidant capacity and, then, assayed both in vitro and in planta for the suppression of Alternaria alternata, the causal agent of early blight of tomato. Ultra high-performance liquid chromatography-diode array detection (UPLC-DAD) analysis showed phenolic profiles of each extract, identifying and quantifying the individual known components, including gallic acid, chlorogenic acid, epicatechin, cumaric acid, rutin, ferulic acid and luteolin. The phenolic-rich extracts of all peppers varieties proved to be active against in vitro mycelial development of A. alternata. Dipping artificially infected tomato leaves using extracts at 10 μg GAE ml−1 of total phenolics, reduced foliar early blight disease severity closely to 50%, on average. The role played by the phenolic complex in the antifungal activity and in disease control efficacy of the extracts, has been discussed.

Keywords: Alternaria alternata, antioxidant activity, disease management, phenolic compounds, phytochemicals.

Early blight is a serious disease of tomato caused by the fungus Alternaria alternata (Fr.) Keissler, which can infect all above ground parts of the plant, causing well demarcated necrotic lesions on leaves, stems and/or fruits. Severe infections, occurring under conducive environmental conditions, can even cause defoliation with losses of net photosynthetic surface area. Treatments with synthetic fungicides are often the sole strategy adopted with trust by growers to counteract the occurrence of dangerous epidemics generating economically significant yield losses. However, public concerns about the negative impacts on the environment and human health due to the use of chemicals in agriculture, are stimulating restrictive policies on their uses. Therefore, the investigations of alternative natural products for plant disease control are becoming necessary. The research and development of plant-based tools effective against fungal pathologies, may have interesting perspectives on sustainable and non-chemical disease management systems. Crude plant extracts that exhibit antimicrobial activity deriving from the carried phytochemicals, have a great potential for eco-friendly antifungal applications: that is why they are receiving increasing attention in the last times. Plants produce a broad range of secondary metabolites, including phenolics, notably involved in the plant defence mechanisms. They are toxic to pathogens (Bennett and Wallsgrove, 1994) and can be pulled out by selective solvent-extraction processes in crude concentrated bioactive extracts (Gurjar et al., 2012; Pane et al., 2013).

The efficacy of plant extracts has widely been reported mainly in counteracting post-harvest biotic injuries (Da Cruz Cabral et al., 2013), while fewer studies have been published on the control of aerial plant diseases. Thangavelu et al. (2013), for example, showed that spraying banana plants with Zimmu water extract reduced severity of leaf spot disease caused by Mycosphaerella eumusae. While, a comprehensive screening of methanolic extracts of 183 plant materials for in vivo activity on six pathosystems, allowed to select those from Achyranthes japonica and Rumex crispus as the most effective in controlling powdery mildew of cucumber caused by Sphaerotheca fuliginea (Kim et al., 2004).

Two recent studies showed the efficacy of foliar wild pepper phenolic-enriched extracts to suppress A. alternata both in vitro (Pane et al., 2015) and in vivo in post-harvesting on cherry tomatoes (Pane et al., 2016), opening the debate on the role of the extracted phenolic compounds...
Duncan's test (cate significant differences among bars according to ANOVA, A. alternata methanolic solution (50% vol.) for 24 h at room temperature. Each material, was extracted by soaking in 100 ml hydro-methanolic foliar extracts was assessed, both tifungal activity. In particular, the suppressiveness of the extracts was assessed, both in vitro and in planta, against 2,2-diphenyl-1-picrylhydrazyl (DPPH) ± standard deviation (n = 3), in the different hydro-methanolic foliar extracts of Capsicum annuum L. samples, Cazzzone Giallo (CG), Cazzzone Rosso (CR), Friariello Napoletano (FN), Marconi Giallo (MG), Marconi Rosso (MR), Sassaniello Giallo (SG) and Sassaniello Rosso (SR). Total phenolics are expressed as μg Gallic Acid Equivalents (GAE) ml−1. Different letters indicate significant differences among bars according to ANOVA, Duncan’s test (p level ≤ 0.05).

in the observed phytoiatric activity. These phytochemical models induced also to explore pepper niche crops, such as pepper local landraces diffuse in Campania Region (Italy), as source of extracting antifungal botanicals in the view of valorisation of their co-products.

The current work aims to investigate the phenolic profile of the hydro-methanolic foliar extracts of Capsicum annuum cultivated genotypes and their implications on antifungal activity. In particular, the suppressiveness of the extracts was assessed, both in vitro and in planta, against A. alternata under laboratory conditions. Plant materials used in this study were seven foliar samples of local landraces of Capsicum annuum L., including “Cazzzone Giallo” (CG), “Cazzzone Rosso” (CR), “Friariello Napoletano” (FN), “Marconi Giallo” (MG), “Marconi Rosso” (MR), “Sassaniello Giallo” (SG) and “Sassaniello Rosso” (SR), collected on a raw of ten plants each at an experimental site of Scafati (Salerno, Italy). A representative sample of 10 g dried (70°C) and powdered leaves for each material, was extracted by soaking in 100 ml hydro-methanolic solution (50% vol.) for 24 h at room temperature. Thus, extracts were filtered (Whatman No. 1 filter paper), centrifuged (10,000 g, 2 min) and vacuum-dried (rotary evaporator at 30°C) to remove the solvent. The residues were re-suspended in distilled water 10% of the initial volume to obtain the highest concentrated extract batches, which, after filter-sterilization (filter 0.22 μm pore size), were stored at −20 °C until use.

Phenolic composition and antioxidant activity of extracts were assessed in the concentrated batches. The colorimetric analysis of total phenolic content of the extract was determined following the method of Singleton and Rossi (1965). Since the quantification was based on a standard curve generated using gallic acid, results were expressed as μg of gallic acid equivalents (GAE) ml−1 of the extract. The chromatographic analysis was performed in ultra high-performance liquid chromatography-diode array detection (UPLC-DAD) by an ACQUITY Ultra Performance LC™ system linked to a photodiode array detector (PDA) 2996 detector (Waters, Milford, MA, USA) following the method reported in Fratianni et al. (2011) and Pane et al. (2016). The phenolic compounds were identified both qualitatively and quantitatively comparing the peak areas on the chromatograms of samples with those of different known standards (gallic acid, chlorogenic acid, epicatechin, p-coumaric acid, rutin, ferulic acid and luteolin) previously diluted in methanol.

Free radical scavenging activity of the extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand-Williams et al., 1995). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma Aldrich, Germany) dissolved in methanol (5 μg/ml) was used for a calibration curve of DPPH reduction and as a chemical reference in comparison to the antioxidant capacity of the extracts. The analyses were performed in three independent experiments.

A high virulent isolate of the pathogen Alternaria alternata, maintained on potato dextrose agar (PDA, Difco) at 25 °C, was used in this study. The inhibitory effects of the extracts on the in vitro growth of the fungus were assessed by pouring each extract into a 0.1× Czapek Dox solid medium (Czapek Dox Broth 3.4 g l−1, agar 15 g l−1) culture into Petri dishes (3 cm diameter), to reach the final concentrations of 1, 10 and 100 μg of total phenolics (TP) ml−1. Then, plates were inoculated in triplicate with a fungal plug (0.5 cm diameter) cut from a Czapek growing sub-culture, and incubated in the dark at 25°C. The radius of each fungal colony was measured when the fungi reached the edge of the not amended control plates. The percentage of fungal growth under the treatments was calculated with the formula: Mycelial Growth = 100 × [radius on the treatment/radius on the control]. The experiment was carried out twice.

The in planta control of Alternaria early blight disease by treatments with extracts was evaluated on tomato plantlets cv. Corbarino as described previously by Pane and Zaccardelli (2015) with some modifications. Thus, 20μl of the extract (10 or 100μg of TP ml−1) were pipetted onto each leaf in correspondence of the inoculating point,
while water alone was applied to infected control leaves. After air-drying of about 40 min, plants were infected by inoculating a drop (5 μl) of *A. alternata* spore suspension (1 × 10^5 conidia ml⁻¹) on treated points previously micro-wounded. Then, plants were kept into a plastic chamber (> 98% relative humidity) and incubated in a climatic room at 25°C. The experimental design included seven treatments with extracts and one non-treated control with ten replications (plants) each, arranged in a randomized complete block. After one week, the diameter of the lesions was measured and expressed as percentage of those recorded on the infected controls. The experiment was carried out twice.

Data of *in vitro* and *in planta* experiments were subjected to analysis of variance (ANOVA) and means were separated by Duncan’s test (*P* ≤ 0.05). Since no significant experiment effect was found, data from repeated experiments were combined for the analysis. The percentage data were angular transformed by arcsine root square before undergoing statistical analysis; non-transformed means are shown.

All produced extracts were similar for total phenolic content (Fig. 1A). The levels of these compounds ranged between 652.88 and 755.08 μg GAE ml⁻¹, detected in the lowest (MR) and the largest (SG) concentrated samples, respectively. The DPPH free radical scavenging activity proved to be significantly highest in MR, SG and SR extracts (3.8 μl, on average), while it was lowest in CG, CR and MG samples (4.3 μl, on average). FN sample showed intermediate values of antioxidant activity (Fig. 1B). The profiling of phenolic compounds carried in pepper extracts indicated a slight variability in composition and relative abundance of known constituents. UPLC-DAD analysis revealed the presence of gallic acid, chlorogenic acid, epicatechin, p-coumaric acid, rutin, ferulic acid and luteolin by comparing chromatograms to the retention times of the peaks of the UV spectra of the standards and quantizing against respective calibration curves (Fig. 2). The amount of each of these molecules contained in the extracts was indicated in Table 1. The known phenolics accounted only for about 30% of the total, on average. Gallic acid, chlorogenic acid, epicatechin and ferulic acid were found in all extracts at levels below 10% of the total phenolics, on average. While, p-coumaric acid was found only in MR and SG samples (0.6% of the total, on average), and rutin accounted for 3.9 and 9% of the total in FN and MG extracts, respectively. Finally, luteolin was detected in all samples, with the exception of CR and MG extracts, in the range 0.28-2.23% of the total.

Natural phenolic compounds represent recurring active ingredients of the phytochemical complexes extracted from plants, that have been widely studied for the sustainable control of plant diseases (Gatto et al., 2011). Previous studies highlighted the involvement of this type of molecules in the mechanisms underlying antifungal properties of *Tephrosia apollinea* extracts against a set of phytopathogenic fungi, including *A. alternata* (Ammar et al., 2013) and in controlling olive knot and crown gall disease by *Lawsonia inermis* extracts (Trigui et al., 2013). Phenolic plant-derived constituents are important phytochemicals widely known for their antimicrobial properties (Fratianni et al., 2007; Martins et al., 2015). The molecular structure including one or more benzene rings with one or more hydroxyl groups variously elaborated with substitutions, such as carboxylic and/or methoxyl, may be linked to their effective toxicity (Alves et al., 2013; Arif et al., 2009). The mode of the antifungal action of phenolics is probably due to their lipophilic characteristics, which cause cell membrane disturbance and oxidative phosphorylation uncoupling, and non-specific interaction with proteins (Yun et al., 2015; da Cruz Cabral et al., 2013; Nazzaro et al., 2013; Pereira et al., 2007).
In this work, the antifungal activity of phenolic-rich extracts of pepper leaves was assessed, at equal concentration of the total phenolics, by using the amended plate technique (Rosado-Álvarez et al., 2014) against \textit{A. alternata} hyphal growth. The \textit{in vitro} experiments indicated that all samples showed significant inhibitory effects on fungal development at the highest concentration of total phenolics tested (100 μg GAE ml\(^{-1}\)). In addition, samples induced similar rate of mycelial growth reduction between them, which varied between 40 and 57% of the control for CG and SR extracts, respectively, at the highest phenolic compound concentration (Fig. 3). In agreement with our findings, Sayago et al. (2012) found that \textit{Chuquiraga atacamensis}, \textit{Parastrephyla phyliciformis} and \textit{P. lepidophylla} extracts were able to significantly inhibit \textit{in vitro Penicillium digitatum} development already at 100 μg ml\(^{-1}\) of total phenolic compounds.

The in planta experiments confirmed the antifungal potential of the phenolic-rich extracts observed on plates. The phytotherapeutic treatments with the pepper-derived products proved able to reduce the Alternaria early blight on tomato leaves (Fig. 4). Disease severity was contained below 60-40% of the non-treated control, on average, with the dose of 10 μg GAE ml\(^{-1}\). SR extract significantly resulted the most efficacy with a reduction of about 65% than the infected control; SG, MR, MG and FN showed disease control rates statistically similar to SR. However, extracts decreased in their ability to control disease symptoms when used at the higher concentration tested (100 μg GAE ml\(^{-1}\)), and the marked differences between the samples disappeared.

In the current work, interestingly, the most active extracts in controlling early blight \textit{in planta} (SR, SG, MR, Mg and FN) resulted among the richest in the phenolic profile (with the exception of SR) and the highest in the antioxidant capacity (with the exclusion of MG). Extracts antifungal properties appear to be slightly linked to the concentration of epicatechin and luteolin, while no clear relationship of the biological effects with the other identified phenolics was found. Findings suggest that the presence of a broader set of phenolic molecules could be

### Table 1. Amount of known phenolic compounds detected in the hydro-methanolic \textit{Capsicum annuum} L. extracts samples, Cazzone Giallo (CG), Cazzone Rosso (CR), Friariello Napoletano (FN), Marconi Giallo (MG), Marconi Rosso (MR), Sassaniello Giallo (SG) and Sassaniello Rosso (SR).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Gallic acid (μg ml(^{-1}))</th>
<th>Chlorogenic acid (μg ml(^{-1}))</th>
<th>Epicatechin (μg ml(^{-1}))</th>
<th>p-Coumaric acid (μg ml(^{-1}))</th>
<th>Rutin (μg ml(^{-1}))</th>
<th>Ferulic acid (μg ml(^{-1}))</th>
<th>Luteolin (μg ml(^{-1}))</th>
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<tbody>
<tr>
<td>CG</td>
<td>31.98</td>
<td>32.37</td>
<td>31.49</td>
<td>−</td>
<td>−</td>
<td>75.66</td>
<td>2.93</td>
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<tr>
<td>CR</td>
<td>26.90</td>
<td>33.27</td>
<td>25.92</td>
<td>−</td>
<td>−</td>
<td>39.35</td>
<td>−</td>
</tr>
<tr>
<td>FN</td>
<td>24.27</td>
<td>54.37</td>
<td>41.03</td>
<td>−</td>
<td>25.69</td>
<td>55.77</td>
<td>1.85</td>
</tr>
<tr>
<td>MG</td>
<td>29.69</td>
<td>31.38</td>
<td>42.87</td>
<td>−</td>
<td>68.54</td>
<td>54.84</td>
<td>−</td>
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<tr>
<td>MR</td>
<td>36.07</td>
<td>70.21</td>
<td>71.25</td>
<td>3.99</td>
<td>−</td>
<td>70.09</td>
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<tr>
<td>SG</td>
<td>35.96</td>
<td>51.47</td>
<td>86.28</td>
<td>4.75</td>
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<td>66.24</td>
<td>16.82</td>
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<tr>
<td>SR</td>
<td>34.58</td>
<td>31.64</td>
<td>49.62</td>
<td>−</td>
<td>−</td>
<td>48.73</td>
<td>2.89</td>
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</table>

Fig. 3. Effect of \textit{Capsicum annuum} L. landraces hydro-methanolic extracts, Cazzone Giallo (CG), Cazzone Rosso (CR), Friariello Napoletano (FN), Marconi Giallo (MG), Marconi Rosso (MR), Sassaniello Giallo (SG) and Sassaniello Rosso (SR), applied at dose of 1, 10 and 100 μg of total phenolics (TP) ml\(^{-1}\), on mycelial growth of \textit{Alternaria alternata}. Values are expressed as percentage with respect to the non-treated plates. Asterisk indicated the significance of the differences of all samples from the control according to ANOVA, Duncan’s test (p level ≤ 0.05).

Fig. 4. Effects of \textit{Capsicum annuum} L. landraces hydro-methanolic extracts, Cazzone Giallo (CG), Cazzone Rosso (CR), Friariello Napoletano (FN), Marconi Giallo (MG), Marconi Rosso (MR), Sassaniello Giallo (SG) and Sassaniello Rosso (SR), applied at dose of 10 and 100 μg of total phenolics (TP) ml\(^{-1}\), on the severity of tomato early blight. Values are expressed as percentage with respect to the lesion on non-treated leaves. Different letters indicate significant differences among bars according to ANOVA, Duncan’s test (p level ≤0.05).
crucial for the overall activity of the phytochemical complex. The occurrence of synergic work between the different ingredients enhancing each specific contributions, may be also hypothesized. Supporting this assumption, Dias et al. (2015) established a positive interaction among the different components of the variable phenolic profiles detected in hydro-methanolic extracts of grape stem concerning the inhibition of intestinal pathogens. However, further investigations to confirm our hypothesis are still necessary. Recently, the effective association between the antimicrobial activity of the phenolic complex and the whole antioxidant activity, has been also observed in extracts of cherry pomace (Kołodziejczyk et al., 2013) and Inula spp. (Gokbulut et al., 2016). Here, the antioxidant properties of extracts showed a possible involvement in counteracting in planta disease progression. Actually, the scavenging of reactive oxygen species generated in plant tissues subsequently to the infective processes of the necrotrophic pathogen A. alternata, could contribute to block the cell death in advancing and the spread of the lesions (Taheri et al., 2014). However, this hypothesis has also to be confirmed by other studies.

This work explored the phytochemical character of extracts of cultivated pepper leaves and showed their potential in suppressing the in vitro and the in planta development of the tomato pathogen, A. alternata. The relevance of phenolic profiles and the radical scavenging activity of the treatments has been discussed, providing new insights about the topic of botanicals applied to the sustainable plant disease management. Our results suggest the potential of the co-products by the tested pepper landraces to be recycled and valorised through sustainable plant disease management applications.

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