



Control of isolates of *Fusarium* species by using extracts of *Rubus ulmifolius* micropropagated plantlets

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Abstract

We developed a micropropagation protocol to obtain *in vitro* plantlets from nodal explants of *Rubus ulmifolius*. The methanolic extract of dried plantlets was assayed for antifungal activity on the growth of eight isolates of *Fusarium* species. The molecular components of the extract, separated by column chromatography, were analysed by HPLC. *Rubus ulmifolius* micropropagated plantlets provide uniformly conditioned material available all year around. Their phytochemicals are characterized by a high antioxidant capacity, water-solubility and ability to inhibit the growth of the eight isolates of *Fusarium* species.

Key words: *Rubus ulmifolius*, micropropagated plantlets, secondary metabolites, antifungal activity, isolates of *Fusarium* species.

Introduction

Fusarium species are common contaminants, which grow on many herbaceous plants, various fruits, cereals and stored vegetable products ¹. To control these infections, many synthetic products have been used, often in excessive amounts. This has induced resistance in fungal pathogens and accumulation of residues in plants ², soil ³, water ⁴ and animals ⁵. The application of antifungal substances of natural origin is receiving increasing attention since it could represent an alternative to synthetic biocides. The "*in vitro*" culture of plants represents a suitable tool to obtain standardized extracts from plant material of the same genotype grown in uniform conditions and available year round. The extracts obtained from such plant tissues do not contain pollutants, herbicides or pesticides and, in the case of wild plants, the ecosystem is not modified by the intensive harvesting.

Rubus ulmifolius (Rosaceae) is a perennial shrub distributed throughout Italy from 0 to 1100 m above sea level. In folk medicine, its leaves are used internally for intestinal inflammations ⁶. Both leaves and fruits showed a high concentration of vitamin C, anthocyanins along with significant amounts of flavonoids, such as quercetin and kaempferol, and phenolic acids, such as caffeic acid and chlorogenic acid ^{7,8}. In addition, extracts obtained from different species of *Rubus* showed antimicrobial activity on fungi and bacteria ^{9,10}.

We report on the phenolic composition and antioxidant capacity of an extract from micropropagated plantlets of *Rubus ulmifolius* as well as its inhibitory activity on the growth of isolates of eight phytopathogenic *Fusarium* species with the aim of obtaining a natural antifungal product.

Experimental

Plant material and *in vitro* culture: Shoots were induced from nodal explants taken from apical portions of young branches of *Rubus ulmifolius* Schott, with no more than 10 buds, collected in the Urbino area (Marche, Central Italy) at 500 m above the sea level. Explants were cultured in Murashige and Skoog medium ¹¹, supplemented with 3% sucrose, 0.8% agar and different concentrations of N⁶-benzyladenine (BA) and naphthaleneacetic acid (NAA) (multiplication phase; MP). Explants were incubated in a growth chamber at 25±2°C in the light (cool white fluorescent light at 50 µmol m⁻² s⁻¹) under a 16-h photoperiod. The newly formed shoots were dissected and transferred to the same medium supplemented with 0.1 or 1 mg/l BA to promote growth of shoots (elongation phase; EP). Finally, elongated shoots were transferred to half strength MS medium with or without auxin, NAA or indolebutyric acid (IBA) at 0.1 or 1 mg/l to initiate root formation (rooting phase; RP). Plantlets were maintained for one month in a growth chamber at the same conditions reported above.

Extract preparation: *Rubus* plantlets in MP, EP and RP were collected and shade dried at room temperature. The crude methanolic extract was prepared by extracting 40 g of the dry ground material in 100 ml of methanol three times in 24 h according to McCutcheon *et al.* ⁹. The crude methanolic extracts were filtered through a Büchner funnel using a no. 4 filter paper. The filtrate was evaporated to dryness under vacuum and then reconstituted with 10 ml of methanol. Each ml of concentrated extract was equivalent to 4 g of dry material.

Fractionation on Sephadex LH-20: The *Rubus* plantlets in the RP (30 days old) were used to prepare the methanolic extract as reported above. The extract was diluted 1:5 v/v with water and loaded on a Sephadex LH-20 glass column equilibrated with 20% methanol. The column was washed with 20% methanol, followed by 60% methanol and finally by 40% acetone. Fractions (10 ml) were collected; absorbance at 280 nm and total phenols were determined to obtain the elution profile. Six peaks of fractions were collected and indicated as P1- P6; each one was evaporated to dryness on a rotary evaporator to 10% of the initial volume and finally diluted with water to obtain the appropriate concentrations of the working solutions.

Secondary metabolites detection: Total phenolics were determined with Folin-Ciocalteu reagent according to the method of Singleton *et al.*¹² and values were expressed as mg/g gallic acid equivalents. Flavonoids were detected by the method of Eberhardt *et al.*¹³. Analysis of phenolic fractions obtained from the Sephadex LH-20 column was performed by HPLC. The HPLC column was a 300 mm x 150 mm RP-18 (Waters); the mobile phase was water (A) vs. methanol (B). Samples were dissolved in methanol and 10 ml of this solution injected in the column. The eluted phenolics were detected by using an UV diode array detector. Identification of individual phenolic compounds was performed using their retention times and spectroscopic data in comparison with commercial standards. The marker standard compounds used in the analysis were: caffeic acid, caftaric acid, chicoric acid, chlorogenic acid, gallic acid, quercetin-3-glucoside, quercetin-3-rhamnoside and kaempferol-3-glucoside.

ORAC assay and protein determination: Oxygen radical absorbance capacity (ORAC) was measured following procedures¹⁴ previously described using a Fluostar Optima plate reader (PBI International, Milan). Protein content was measured with the absorbance at 280 nm.

Antifungal screening: The methanolic extract from micropropagated *Rubus ulmifolius* plantlets in rooting phase was tested against eight phytopathogenic strains of *Fusarium* genus: *F. culmorum* (Smith) Saccardo (ATCC 12656), *F. graminearum* Schwabe (ATCC 15624), *F. poae* (Peck) Wollenweber (ATCC 24383),

F. avenaceum (Corda) Saccardo (ATCC 24362), *F. equiseti* (Corda) Saccardo (ATCC 11853), *F. semitectum* Berkeley et Ravenel (ATCC 15659), *F. sporotrichioides* Sherbakoff (ATCC 24630) and *F. oxysporum* Schl. kindly provided by Dipartimento di Protezione e Valorizzazione Agroalimentare, University of Bologna, Italy. The antifungal activity of the extract was tested by the disc diffusion method¹⁵ according to Jorgensen *et al.*¹⁶ in sterile Potato Dextrose Agar (PDA; Sigma) plates. Paper discs (5 mm diameter) were imbibed with 25, 50 or 100 ml of crude methanolic extracts or Sephadex-fractions or methanol alone (control) and allowed to evaporate at room temperature. Inoculated plates were incubated at 20°C for 36 h in the dark. For each extract or fraction, 4 replicate trials were conducted against each *Fusarium* strain. Nystatin (50 µg) was used as a positive control and methanol as a negative solvent control.

Antifungal activity was evaluated as the average diameter of the inhibition zone surrounding the discs. A clearing zone of 8 mm or greater was used as the criterion for designating significant antifungal activity. The numerical breakdown of the classification was as follows: (-), no activity; (0-1), questionable activity; (1 +), 8.1-10 mm halo diameter; (2 +), 10.1-15 mm halo diameter; (3 +), 15.1-20 mm halo diameter; (4 +), 20.1-25 mm halo diameter; (5 +), > 25 mm halo diameter. Both the whole extract and the chromatographic fractions of *Rubus ulmifolius* were tested at the same total phenol concentrations; we used 25, 50 and 100 µl of the working solution, corresponding to 0.9, 1.8 and 3.6 mg of phenols, respectively.

Results and Discussion

To detect the growth phase able to provide the maximal phenolic content and antioxidant capacity, we compared methanolic extracts obtained from micropropagated shoots in the multiplication (MP), elongation (EP) and rooting phase (RP). The three stages of growth, MP, EP and RP exhibited, on fresh weight (FW) base, a similar content in total phenols (7.1±0.8 mg g⁻¹ FW) and flavonoids (2.2±0.2 mg g⁻¹ FW). The ORAC value of plantlets in the RP was 111±9 µmol TE g⁻¹ FW, a value not significantly different from that of MP and EP. On this basis, to prepare the methanolic extract to be tested as an antifungal agent, we used plantlets in the RP, which provided the highest amount (at least 30% more) of vegetable material. The concentrated methanolic extract, obtained

from dried plantlets in the RP, was equivalent to 4 g of dry material per ml of methanol. In these conditions, the extract showed a phenolic concentration of 36 mg ml⁻¹ and about 1200 ORAC µmol TE ml⁻¹ of ORAC.

The *Rubus* plantlet extract was tested for antifungal activity against eight isolates of *Fusarium* species. Table 1 shows that all isolates of *Fusarium* resulted susceptible to 50 and 100 µl of extract, corresponding to 1.8 and 3.6 mg total phenols, respectively. The highest inhibitory effect was exhibited at 3.6 mg of total phenols against *F. poae*, *F. sporotrichioides* and *F. oxysporum* as revealed by the disc diffusion

Table 1. Antifungal activity of methanol extract obtained from *Rubus ulmifolius* dry plantlets in the rooting phase.

<i>Fusarium</i> species	Inhibition zone				
	Nystatin dihydrate (50 µg)	Methanol control (50 µl)	<i>Rubus</i> extract (25 µl)	<i>Rubus</i> extract (50 µl)	<i>Rubus</i> extract (100 µl)
<i>F. culmorum</i> (Smith) Saccardo (ATCC 12656)	5+	-	-	2+	3+
<i>F. graminearum</i> Schwabe (ATCC 15624)	5+	-	-	2+	3+
<i>F. poae</i> (Peck) Wollenweber (ATCC 24383)	5+	-	-	3+	4+
<i>F. avenaceum</i> (Corda) Saccardo (ATCC 24362)	5+	-	-	2+	3+
<i>F. equiseti</i> (Corda) Saccardo (ATCC 11853)	5+	-	-	2+	3+
<i>F. semitectum</i> Berkeley et Ravenel (ATCC 15659)	5+	-	-	2+	3+
<i>F. sporotrichioides</i> Sherbakoff (ATCC 24630)	5+	-	-	3+	4+
<i>F. oxysporum</i> Schl. ^a	5+	-	-	3+	4+

^akindly provided by Dept. of Protezione e Valorizzazione Agroalimentare, University of Bologna. The results of halo diameter (mm) are means of four replicates. A clearing zone of 8 mm or greater was used as the criterion for designating significant antifungal activity. The numerical breakdown of the classification was as follows: (-), no activity; (1 +), 8.1-10 mm; (2 +), 10.1-15 mm; (3 +), 15.1-20 mm; (4 +), 20.1-25 mm; (5 +), > 25 mm.

method, where the halo diameter of growth inhibition ranged from 20.1 to 25 mm.

To determine the phenolic components, which are mainly responsible for the antifungal activity, the methanolic extract of *Rubus* was fractionated by low pressure column chromatography on Sephadex LH-20. Fig. 1 shows the six constituent peaks of the elution profile, indicated as P1-P6, obtained by changing three eluting solvents. It is worthy of note that most of the proteins are distributed in P1 and P2, while the other peaks are almost free of proteins. Each fraction associated with a peak was evaporated on a rotary evaporator to 10% of the initial volume and finally diluted with water to obtain the appropriate concentrations of the working solutions.

Table 2 shows the characterization of the six fractions as far as the phenols, flavonoids, ORAC units and HPLC data are concerned. P2, P5 and P6 showed the greatest phenolic content and antioxidant capacity. The main compound detected in P1 was chlorogenic acid, while P2 contained mainly flavonoids and, in addition, a limited amount of caffeoylquinic acids. P3 contained caftaric acid and a minor amount of caffeoyl derivatives, while P4, P5 and P6 were rich of tannins both in the hydrolyzable and condensed form.

Table 3 shows the antifungal activity of the six fractions tested at 0.9 and 1.8 mg phenol concentration, as described above for the whole extract. Except fraction P2, which was ineffective, all the other fractions showed antifungal activity with P6 expressing the highest effect. When mixed together P4, P5 and P6 (0.9 mg total phenolic concentration in 25 μ l) determined a dramatic inhibition of the growth of all *Fusarium* isolates. This indicates that most of the antifungal effect of the *Rubus* extract derives from an additive effect of the different endogenous tannins. Fractions P1 and P3 containing chlorogenic and caftaric acid, respectively, showed remarkable antifungal activity, while P2 containing flavonoids was ineffective. Phenolic content of vegetable extracts is reported to be directly proportional to antimicrobial activity¹⁷; also the antioxidant capacity has been positively correlated to both antibacterial and antifungal activity, as well¹⁸. The antioxidant capacity detected in our fractions, did not allow us to draw a positive correlation between antioxidant capacity and antifungal effect. In fact, the ORAC/phenol ratios, calculated from the data reported in Table 2, correspond to 21 and 33 for P1 and P2 fractions, which showed weak to no antifungal

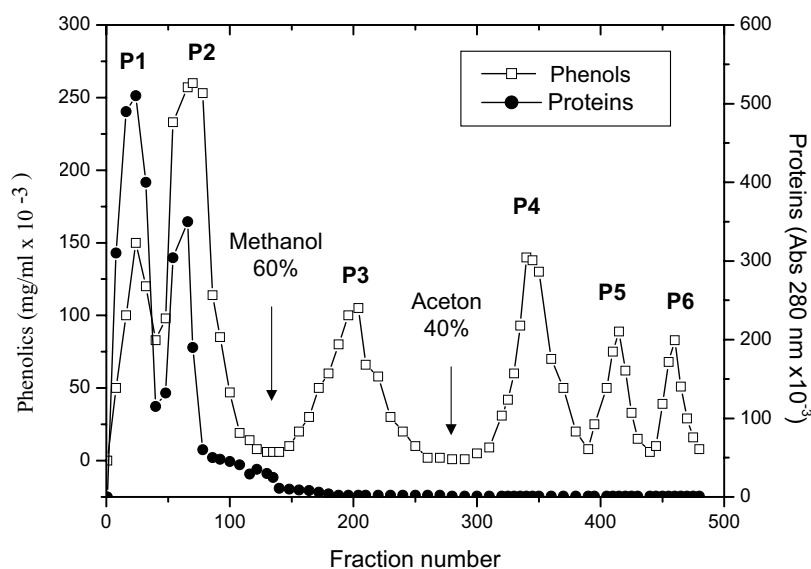


Figure 1. Chromatographic profile of crude methanol extract of *Rubus ulmifolius* on Sephadex LH-20 column. The arrows indicate the fractions where eluting solvents were applied. The fraction volume was 10 ml.

activity, respectively. On the contrary, ratios ranging 5-8 were measured for P3, P4, P5 and P6 fractions, which show a stronger antifungal activity. Therefore, the antifungal activity of *R. ulmifolius* plantlets does not seem to be due to oxidoreductive interactions with proteins or other cellular components but more likely to the interference of phytochemicals with the growth of the fungus.

The antifungal activity of *Rubus* extract had never previously been tested on *Fusarium* isolates. In fact, the only report¹⁰ concerning a methanolic fraction obtained from *R. ulmifolius* dried leaves, branches and flowering tops showed activity against some bacteria and fungi other than *Fusarium*. In a previous paper, McCutcheon *et al.*⁹ tested the methanolic extract of adult leaves of *Rubus parviflorus* against *Fusarium triticum*; the concentration of the extract used in this study corresponded to 0.08 g of dry material and this extract was effective at 1+, corresponding to a 8-10 mm halo diameter of the inhibition zone. Since we tested an extract about three folds more concentrated (0.2 g in 50 μ l) obtaining an inhibitory effect 3+ on the same scale, our results confirm the data of McCutcheon *et al.*⁹ and extend the spectrum of the antifungal effect of *Rubus* species to other isolates of *Fusarium*.

Conclusions

The *Rubus* extract shows a very high antioxidant capacity and antifungal activity. Moreover the extract is water-soluble and does not release strong odours and flavours. After the due tests of toxicity and biodegradability, this extract can be proposed in the control of postharvest diseases of fresh commodities, which are particularly vulnerable to *Fusarium* contamination.

Table 2. Characterization of the eluates from the Sephadex LH-20 column loaded with a whole extract of *Rubus ulmifolius*.

	Volume (ml)	Phenols (mg)	Flavonoids (mg)	ORAC (μ mol TE)	Main compounds	HPLC retention time (min)
Whole extract	30	997	347	12.474		
P1	12	17	8	362	Chlorogenic acid	10.48
P2	22	85	82	1.855	Flavonoids	6.81 40.23
P3	15	31	10	186	Caftaric acid	18.62
P4	15	25	5	201	Tannins	20-28
P5	10	402	51	2.093	Tannins	20-28
P6	16	202	12	1.301	Tannins	20-28

The methanolic extract was loaded on a 5.5 cm x 30 cm Sephadex LH-20 column, equilibrated with 20% methanol. Elution was performed as indicated in Fig. 1. The yields were: 76% phenols; 49% flavonoids; 48% ORAC units. Chlorogenic acid in P1 was 60% of all components; caftaric acid in P3 was 78%. P4, P5 and P6 were 100% tannins in both condensed and hydrolysable forms.

Table 3. Antifungal activity of single fractions of methanolic extract after column chromatography on Sephadex LH-20.

<i>Fusarium</i> species	Nystatin dihydrate 50 µg	Methanol control 50 µl	P1 50 µl	P2 50 µl	P3 50 µl	P4 50 µl	P5 50 µl	P6 50 µl	P4+P5+P6 25 µl
<i>F. culmorum</i>	5+	-	2+	-	2+	1+	2+	2+	5+
<i>F. graminearum</i>	5+	-	2+	-	2+	2+	2+	3+	5+
<i>F. poae</i>	5+	-	2+	-	2+	2+	2+	3+	5+
<i>F. avenaceum</i>	5+	-	2+	-	2+	2+	2+	3+	5+
<i>F. equiseti</i>	5+	-	2+	-	2+	2+	2+	2+	5+
<i>F. semitectum</i>	5+	-	2+	-	2+	2+	2+	3+	5+
<i>F. sporotrichioides</i>	5+	-	3+	-	2+	2+	2+	3+	5+
<i>F. oxysporum</i>	5+	-	2+	-	2+	2+	2+	3+	5+

Fractions were tested at the same total phenol concentrations of the crude extract; we used 25, and 50 µl of the working solution, corresponding to 0.9 and 1.8 mg of phenols, respectively. The results of halo diameter (mm) are means of four replicates. A clearing zone of 8 mm or greater was used as the criterion for designating significant antifungal activity. The numerical breakdown of the classification was as follows: (-), no activity; (1 +), 8.1-10 mm; (2 +), 10.1-15 mm; (3 +), 15.1-20 mm; (4 +), 20.1-25 mm; (5 +), > 25 mm.

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