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# Effects of polysaccharides from *Botryotinia fuckeliana (Botrytis cinerea)* on *in vitro* culture of table and wine grapes (*Vitis vinifera*)

by

G. Fanizza[1]), V. Bisignano[2]), S. Pollastro[3]), M. Miazzi[3]) and F. Faretra[3])

[1]) Istituto di Miglioramento Genetico, Università di Bari, Italia
[2]) Istituto del Germoplasma del Consiglio Nazionale delle Ricerche, Bari, Italia
[3]) Dipartimento di Protezione delle Piante dalle Malattie, Università di Bari, Italia

S u m m a r y : Shoots of several table and wine grape cultivars were cultured *in vitro* on a medium supplemented with polysaccharides obtained from a culture filtrate of *Botryotinia fuckeliana* through differential ethanolic precipitations. The general effects of polysaccharides resulted in leaf yellowness and in a reduction of fresh and dry weight. Differential response of assayed cultivars to polysaccharides seemed to be not related to their bunch susceptibility to grey mould under field conditions.

K e y w o r d s : grapevine, screening test, tolerance, grey mould, polysaccharides.

## Introduction

Grey mould, induced by *Botryotinia fuckeliana* (de Bary) Whetz. (=*Botrytis cinerea* Pers.), causes heavy losses of yield in table and wine grapes in all temperate areas. In Southern Italy, the disease is severe on late ripening cultivars, especially when high relative humidity and raining occur before harvesting time.

Chemical control of grey mould is costly and is often hampered by development of fungicide resistance in the pathogen; in addition, chemical treatments may leave fungicide residues in grapes. Although biological control of grey mould is possible, the use of resistant cultivars might be an attractive alternative. Screening procedures represent a crucial step in any selection method for disease resistance. Tissue culture techniques might be useful for such purpose (Hammerschlag 1984; Daub 1986) because large numbers of genotypes might be screened *in vitro* in a limited amount of space and time.

Several papers have been published on *in vitro* selection of *Vitis* for resistance to pathogens such as *Plasmopara viticola* (Berk. *et* Curt.) Berl. *et* De Toni (Barlass *et al.* 1986), *Uncinula necator* (Schw.) Burr. (Klempka 1984), *Eutypa lata* (Pers.: Fr.) Tul. *et* C. Tul. (Mauro *et al.* 1988) as well as *B. fuckeliana* (Hoos and Blaich 1988).

It is known that *B. fuckeliana* produces extracellular polysaccharides which include glucanes and rhamnogalacto-mannans (Dubourdieu 1978; Montant and Thomas 1978; Pielken *et al.* 1990). They may play a role as elicitors of plant defence responses and seem to be toxic at higher dosages (Dixon and Fuller 1977; Kamoen 1984; Liswidowati *et al.* 1991; Kamoen and Dubourdieu 1990).

Recent results (Vannel *et al.* 1991 a, b; Bessis *et al.* 1992) have suggested the use of culture filtrates and phytotoxic polysaccharides for *in vitro* selection of resistant plants. In this work we have used polysaccharides secreted by *B. fuckeliana* and a large number of grapevine cultivars to evaluate the reliability of such *in vitro* screening test.

## Materials and methods

P l a n t m a t e r i a l : Several table or wine grape cultivars (Tab. 1) with different level of susceptibility to grey mould were cultured *in vitro* on a modified MS medium (Harris and Stevenson 1982). The uppermost 30-35 mm portion of elongated shoots were transferred in vessels containing the same medium, with 1 mg/l BAP and supplemented with polysaccharides obtained from *B. fuckeliana* culture filtrate (see below). Three vessels (replicates), each containing 5 shoots, were used for each cultivar and treatment. They were maintained in a growth chamber at 25 °C with a 16 h photoperiod. Fresh and dry weight of each replicate were measured and leaf colour (SPAD) was evaluated by a leaf greenness meter (SPAD-501, Minolta Corp.) (Marquard and Tipton 1987; Fanizza *et al.* 1991) after 2 months of *in vitro* culture. All data were submitted to statistical analysis.

Shoots of several cultivars were sectioned at different distance from their base by using a cryotome. Sections were mounted in glycerine and observed with a Zeiss Photomicroscope III equipped with epifluorescence system including mercury lamp HBO 100 W/2 and blue-violet filter set (exciter filter BP 436/8, beam splitter FT 460 and barrier filter LP 470).

O b t a i n m e n t o f p o l y s a c c h a r i d e s : Preliminary experiments showed that *B. fuckeliana* monoascosporic strain SAR2116 was a good producer of

Correspondence to: Prof. G. Fanizza, Università di Bari, Istituto di Miglioramento Genetico delle Piante Agrarie, Via Amendola, 165/A, I-70126 Bari, Italy

polysaccharides. The strain was grown on potato dextrose agar for 10 d at 21±1°C and exposed to lighting from a combination of fluorescent and NUV lamps (12 h/d) to obtain conidia. Conidia were scraped from the surface of cultures and suspended in water containing 0.05 % tween 20.Aliquots (10 ml) of the conidial suspension were added to 2 l Erlenmeyer flasks containing 800 ml of Czapek-Dox medium, to give a final concentration of $2.5 \infty 10^4$ conidia/ml medium. The strain was then grown at 21±1°C in still cultures in the darkness for 4 weeks. Culture filtrate was collected and measured after mycelium removal by filtration on Miracloth (Calbiochem). Polysaccharides were obtained following the method of DUBOURDIEU (1978), with minor modifications. The filtrate was adjusted at pH 3 with 1N hydrochloridic acid. Addition of half volume of ethanol caused precipitation of P0.5 fraction (glucanes), which was removed by centrifugation (10,000 rpm, -10 °C, 20 min). The surnatant was then added with further 3.5 volumes ethanol and kept 24 h at 4 °C; the P4 fraction, which consists of rhamno-galacto-mannans was collected by centrifugation as above. Collected fractions were washed with ethanol, dried under vacuum, weighed, dissolved in distilled water, and then dialysed (membrane cut off: $1.2 \infty 10^4$ Da) for 48 h against water. The P4 fraction was then autoclaved before to be added (4 g/l) to modified MS medium, just before dispensing to vessels. VANNEL *et al.* (1991 a) found that the toxicity of culture filtrates is not reduced by autoclaving. The concentration of P4 fraction into the medium was made double of that in the fungal culture filtrate (VANNEL *et al.* 1991 b).

## Results and discussion

Still culture of *B. fuckeliana* strains in large volumes of Czapek-Dox medium allowed only limited fungal growth. In this conditions a small amount of P0.5 fraction (glucanes) and a large amount of P4 fraction (rhamno-galacto-mannans) were secreted by the fungus into the filtrate, as compared to shaken cultures on the same medium. For example, in experimental conditions described above, the strain SAR2116 produced 600-900 mg/l of dried mycelium, 60-100 mg/l of P0.5 and 2-2.5 g/l of P4.

The incorporation of polysaccharides in the growth medium resulted in a decrease of fresh and dry weight of grapevine shoots (Tab. 1) and in a yellowish colour of their leaves (Tab. 2); these effects were statistically significant and a different response was detected among cultivars. Mass decrease due to rhamno-galacto-mannans was more evident in Moscato Reale (92 %) than in Regina Bianca (51 %, Fig. 1). Leaf yellowness was observed in almost all cultivars; it was more pronounced in Trebbiano (SPAD decrease: 63 %), and almost negligible in Copeta (SPAD decrease: 8 %, Tab. 2).

Leaf yellowness was an evident symptom that stands out on in vitro grapevine cultures supplemented with the P4 fraction of polysaccharides. This is in agreement with findings of BESSIS *et al.* (1992), who observed chlorophyll destruction in leaves exposed to culture filtrate of

Table 1

Mean values of fresh and dry (in parenthesis) weight in table (T) and wine (W) grape cultivars and rootstocks (R) after 2 months of *in vitro* culture on a medium supplemented with polysaccharides produced by *Botryotinia fuckeliana* (P4 fraction)

| Cultivar | Control | P4 fraction | Decrease (%) |
|---|---|---|---|
| Malvasia Nera (W) | 15.7 (0.88) ab | 12.6 (0.69) a | 20 (22) |
| Baresana (T) | 19.7 (0.97) a | 11.3 (0.66) ab | 43 (32) |
| Copeta (T) | 18.3 (0.93) a | 7.2 (0.47) abc | 61 (49) |
| Regina Bianca (T) | 12.7 (0.89) bc | 6.2 (0.45) bc | 51 (44) |
| Alphonse Lavallé (T) | 12.9 (0.71) bc | 5.4 (0.38) bc | 58 (46) |
| Uva di Troia (W) | 9.9 (0.60) bc | 4.6 (0.25) bc | 54 (58) |
| Trebbiano Dorato (W) | 8.9 (0.65) c | 4.2 (0.24) bc | 53 (63) |
| Verdeca (W) | 14.9 (0.82) ab | 4.0 (0.20) bc | 73 (76) |
| Inzolia (T) | 12.1 (0.81) bc | 3.0 (0.17) c | 75 (79) |
| Primitivo (W) | 12.0 (0.75) bc | 2.8 (0.14) c | 77 (81) |
| Gloire M. (R) | 7.4 (0.62) c | 2.4 (0.12) c | 68 (81) |
| 140 R (R) | 7.5 (0.61) c | 2.0 (0.11) c | 73 (82) |
| Sangiovese (W) | 8.9 (0.68) c | 1.8 (0.11) c | 80 (84) |
| Bianco di Alessano (W) | 15.4 (0.81) ab | 1.8 (0.10) c | 88 (88) |
| Trebbiano (W) | 12.1 (0.73) bc | 1.4 (0.09) c | 88 (88) |
| Regina Nera (T) | 15.7 (0.73) ab | 1.4 (0.09) c | 91 (88) |
| Regina dei Vigneti (T) | 10.8 (0.53) bc | 1.2 (0.08) c | 89 (85) |
| Bombino Bianco (W) | 9.0 (0.60) c | 0.8 (0.06) c | 91 (90) |
| Negroamaro (W) | 6.6 (0.47) c | 0.7 (0.05) c | 89 (89) |
| Moscato Reale (W) | 6.6 (0.48) c | 0.5 (0.05) c | 92 (90) |
| Total mean | 11.9 (0.71) | 3.7 (0.22) ** | 69 (69) |

Means (fresh weight) followed by same letters are not significantly different at P=0.01 level (Newman-Keuls test)
** P=0.01 between treatments (t-test).

Table 2

Mean values of SPAD (leaf greeness) in table (T) and wine (W) grape cultivars and rootstocks (R) after 2 months of *in vitro* culture on a medium supplemented with polysaccharides produced by *Botryotinia fuckeliana* (P4 fraction)

| Cultivar | Control | P4 fraction | Decrease (%) |
|---|---|---|---|
| Baresana (T) | 18.0 a | 16.0 a | 11 |
| Malvasia Nera (W) | 14.6 ab | 12.6 ab | 14 |
| Negroamaro (W) | 19.0 a | 12.0 abc | 37 |
| Copeta (T) | 12.5 abc | 11.5 abc | 8 |
| Primitivo (W) | 18.6 a | 10.0 abc | 46 |
| Moscato Reale (W) | 17,2 ab | 10.0 abc | 42 |
| Regina Nera (T) | 17.0 ab | 10.0 abc | 41 |
| Bianco di Alessano (W) | 16.3 ab | 8.6 bc | 47 |
| Regina Bianca (T) | 13.0 abc | 8.5 bc | 35 |
| Inzolia (T) | 10.6 bc | 7.0 bc | 34 |
| Alphonse Lavallé (T) | 15.3 ab | 7.0 bc | 54 |
| Verdeca (W) | 13.0 abc | 7.0 bc | 46 |
| Trebbiano (W) | 19.0 a | 7.0 bc | 63 |
| Bombino Bianco (W) | 10.6 bc | 6.0 bc | 43 |
| Gloire M. (R) | 9.2 c | 6.0 bc | 35 |
| Uva di Troia (W) | 12.6 abc | 6.0 bc | 52 |
| Sangiovese (W) | 10.5 bc | 6.0 bc | 43 |
| Regina dei Vigneti (T) | 9.0 c | 5.5 bc | 39 |
| 140 R (R) | 11.6 bc | 5.0 bc | 57 |
| Trebbiano Dorato (W) | 8.0 c | 4.3 c | 47 |
| Total mean | 13.8 | 8.3** | 40 |

Means followed by same letters are not significantly different at P=0.01 level (Newman-Keuls test)
** P=0.01 between treatments (t-test).

*B. fuckeliana* The mechanism through which polysaccharides causes leaf colour degradation is not known. The leaf color change is a symptom of the beginning of leaf senescence, a phenomenon which may be caused by different factors which induce stress (abiotic or biotic). The influence of nutrients and cytokinins on leaf senescence has been reported by different authors (RICHMOND and LANG 1957; THIMANN 1987; SIGN *et al.* 1992). Thus, leaf yellowness in grape shoots might be due either to direct toxicity

of P4 fraction or to interactions of polysaccharides with cytokinins contained in the culture medium.

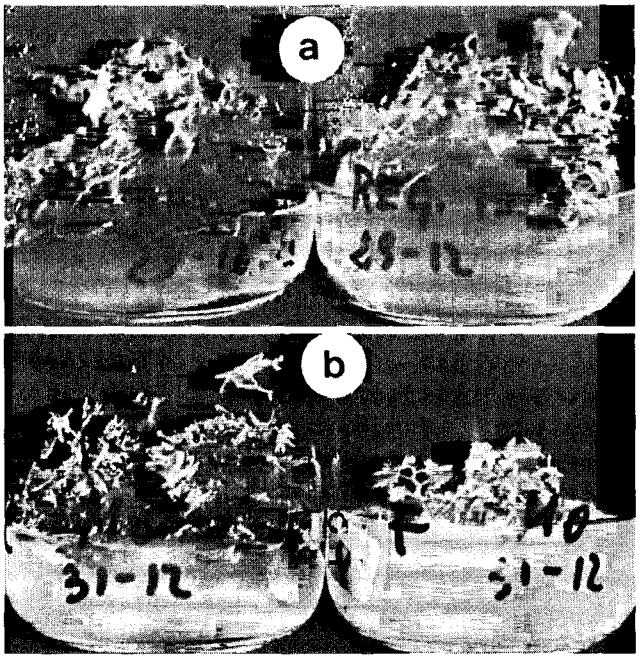


Fig. 1: Shoot growth of Regina Bianca (a), and Moscato Reale (b) after two months of *in vitro* culture on control medium (left) and medium supplement with 4 g/l P4 fraction (right).

Observations at the microscope of cross sections from stunted shoots grown on medium containing the P4 fraction revealed that large parts of tissues were brownish in colour. Tissue browning was limited to the downmost part of shoots at direct contact with the medium. Brown tissues, but not healthy ones, emitted a strong yellow autofluorescence when excited with blue-violet light (Fig. 2). This indicates that the P4 fraction probably induced accumulation of phenolic substances, which are well known for their role in defence mechanisms (LANGCAKE and PRYCE 1976).

Our results indicate that the P4 fraction of culture filtrate from *B. fuckeliana* causes leaf yellowness and growth reduction *in vitro* cultures of grapevines, with a different response among cultivars. Similar results were previously obtained by VANNEL *et al.* (1991 a, b) and BESSIS *et al.* (1992), who suggested that a bioassay based on differential response of grapevines to P4 fraction might be exploited to screen *in vitro* cultivars tolerant to grey mould. In our research, screening a larger number of genotypes, we have detected a low relationship between the cultivar response *in vitro* and their susceptibility to grey mould under field conditions; for instance some cvs such as Baresana and Moscato Reale are both highly susceptible to *B. fuckeliana* under field conditions, as it is well known, but *in vitro* they showed a different response. In fact the cv. Baresana presented little or no sensitivity while the cv. Moscato Reale high sensitivity to P4 fraction (Tab. 1).

Further and more extensive investigations are suggested to carry out *in vitro* and field experimental conditions in order to evaluate the possibility to apply the *in vitro* culture as a screening test for the tolerance to *B. fuckeliana* in table and wine grapes.

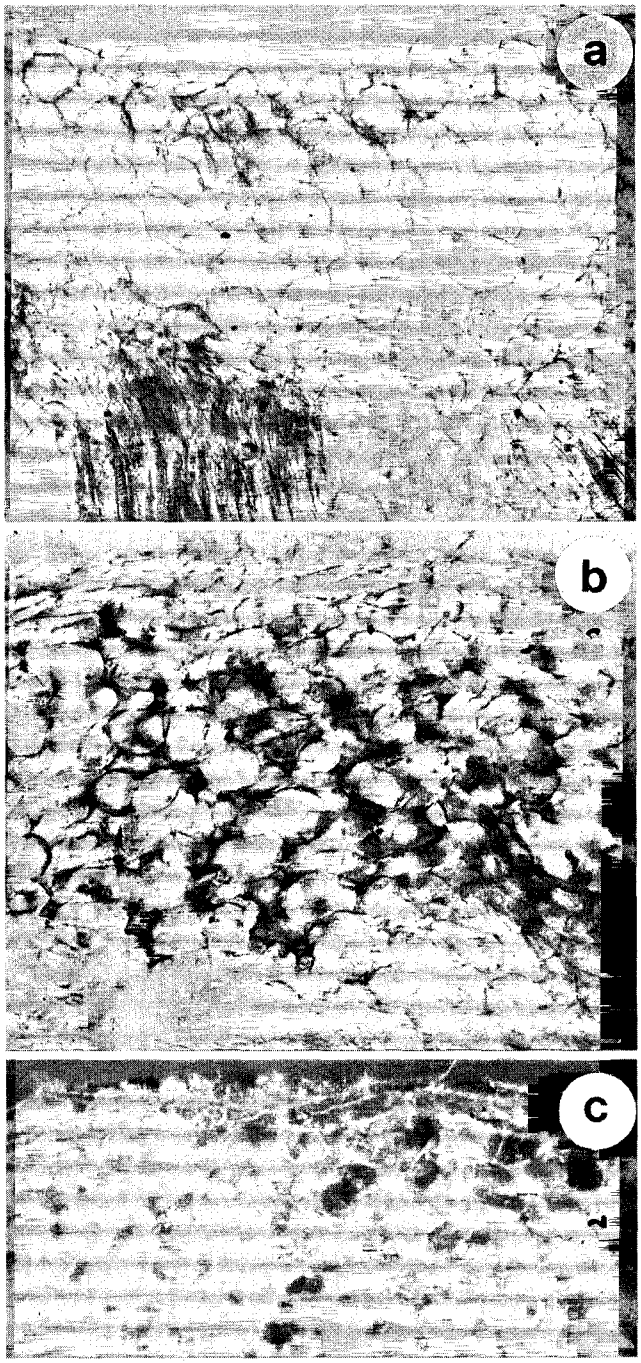


Fig. 2: Cross sections from basal parts of shoots (Regina Bianca) grown on control medium (a) and on medium added with 4 g/l P4 fraction (b). Browned tissues in (b) emitted a yellow autofluorescence when exited by blue-violet light (c).

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