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Detrimental effect of quercetin on phytoplasma-infected *Catharanthus roseus* (L.) G. Don shoots grown in vitro

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Quercetin is known to possess antimicrobial activity against bacteria, fungi, and viruses. The activity of this flavonoid against phytoplasmas, non-cultivable plant pathogenic bacteria that cause numerous plant diseases, has never been examined before. The aim of this research was to examine the effect of different concentrations of quercetin (10 μM, 100 μM and 1 mM) on '*Candidatus* Phytoplasma asteris' and on phytoplasma-infected periwinkle shoots grown in vitro. The addition of quercetin neither supported the growth of the shoots nor induced the remission of symptoms in the infected plants. On the contrary, addition of quercetin induced browning of leaves and the appearance of black spots on the leaves of treated infected and non-infected shoots. It also had no curative effect against the pathogen. Phytoplasma presence was confirmed by nested PCR in infected shoots treated with quercetin through three subcultures.

Key words: phytoplasma, bacteria, antimicrobial activity, flavonoid, quercetin, periwinkle, plant tissue culture

Introduction

Flavonoids, natural substances present in all vascular plants, possess an antimicrobial activity against different bacteria, fungi, and viruses (HARBORNE and WILLIAMS 2000, CUSHNIE and LAMB 2005). When the antibacterial properties of flavonoids are discussed, most of the data come from in vitro studies of human pathogenic bacteria. Those studies revealed that many different flavonoids, including quercetin, inhibit bacterial growth. However, different studies on the same bacterial species produced ambiguous results, possibly due to the different inoculum sizes and different assays used (disk diffusion assay, broth macrodilution assay, broth microdilution assay, agar well diffusion assay, agar dilution assay) as well as to different diffusion rates of various flavonoids, formation of precipitates or salts and structural alternations of flavonoids inside the applied inoculums. Whether flavonoids act as bacteriostatic or bactericidal agents is under debate because some research groups have shown that flavonoids reduce the number of colony-forming units, while others speculate that this is the consequence of the formation of bacterial aggregates under flavonoid-exposure rather than bactericidal effect (CUSHNIE and LAMB 2005). Several

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hypotheses have been proposed for the mechanisms of the antibacterial action of flavonoids: intereference with DNA synthesis (PLAPER et al. 2003), inhibition of membrane function and inhibition of energy metabolism (CUSHNIE and LAMB 2005).

Data on flavonoid activity against plant pathogens encompass mostly antifungal and antiviral activity. Flavonoids are able to suppress fungal infection or inhibit spore germination, thus protecting plants from fungal attacks (HARBORNE and WILLIAMS 2000). Their antiphytoviral activity encompasses reduction of virus infectivity by interfering with initiation of virus infection (RUSAK et al. 1997), weakening of interactions among coat-protein subunits (VERMA 1973, MALHOTRA et al. 1996) or altering interactions between viral nucleic acid and capside proteins (FRENCH et al. 1991).

Activity of quercetin against phytoplasmas, plant pathogenic bacteria, has not hitherto been tested. Phytoplasmas are wall-less prokaryotes that have a two-host cycle involving plants and insect vectors (CHRISTENSEN et al. 2005). These uncultivable bacteria are the main cause of many economically important diseases infecting several hundred plant species worldwide. Different techniques and treatments have been applied in attempts to cure diseases caused by different phytoplasma species: treatments with tetracyclines, β-amino--butyric acid, polyamines, terpenes, auxines, tissue culture and/or heat or hot water treatment (ĆURKOVIĆ PERICA and ŠERUGA MUSIĆ 2005, ĆURKOVIĆ PERICA 2008a).

The aim of this research was to test whether quercetin treatment could eliminate phytoplasmas from the host or at least induce recovery of phytoplasma-infected plants.

Material and methods

Material and plant tissue culture methods

Catharanthus roseus (L.) G. Don shoots infected with '*Candidatus* Phytoplasma asteris' (strain HYDB), belonging to the 16SrI-B subgroup, were grown in vitro on MS (MURASHIGE and SKOOG 1962) basal nutrient medium supplemented with 100 mgL^{-1} myo--inositol, 1 g L^{-1} casein hydrolysate, 30 g L^{-1} sucrose, 9 g L^{-1} agar and 0.5 mg L^{-1} benzylaminopurine (BA). In such a system, phytoplasmas are present in a high titer and all shoots express symptoms of infection (proliferations, internode shortening, stunting). Phytoplasma-infected shoots were obtained from Phytoplasmology Laboratory of the University of Bologna (IRPCM 2004). Healthy in vitro-grown *C. roseus* shoots were also included in experiments as controls. Each shoot was grown in a test tube (20 × 150 mm) filled with 15 mL of MS nutrient medium. The pH of the medium was adjusted to 5.7 before autoclaving at 118 kPa and 120 °C for 20 min.

In order to test the effect of quercetin (Sigma) on '*Ca.* P. asteris' – infected and healthy shoots, they were transferred to the medium mentioned above, which did not contain agar. This medium was, after autoclaving, supplemented with 10 μM, 100 μM or 1 mM of quercetin. Quercetin stock solutions were prepared in ethanol. Therefore, in the control experiment, ethanol without quercetin was also added to the medium in order to rule out the possibility of the antiphytoplasmal activity of the alcohol. Untreated infected and non-infected controls were also transferred to liquid medium. The cultures were incubated at 22±2 °C under a 16-h photoperiod and subcultured in a 3 week-culture period. Twenty-four infected *C. roseus* shoots per treatment, positive control (untreated infected shoots on the medium with BA), non-infected treated shoots, and negative control (non-infected shoots

on the medium with BA) were included in the experiment (Tab. 1). Shoots were placed on paper bridges with their stems 2–3 mm immerged in the liquid medium Weight (g) and length (cm) were measured on 16 shoots per treatment to insure availability of plant material for phytoplasma detection. Measurements were performed at the beginning and at the end of the first three subcultures. Fresh weight increase and shoot length increase were calculated as a ratio of the final and the initial fresh weight or shoot length, respectively. Mean values, analysis of variance and Duncan's test were used for analysis and interpretation of the data.

Phytoplasma detection

Eight samples (0.5 g each) of randomly chosen infected shoots treated with different concentrations of quercetin, positive control and non-infected shoots were taken after the 3rd subculture in order to be tested for the presence of '*Ca.* P. asteris'. Exceptions were infected shoots grown on 1 mM quercetin, which started to decay in the first subculture and were therefore tested after the first subculture. The procedure for phytoplasma detection, including total nucleic acid isolation, PCR amplification and product analysis was previously described (Ćurković Perica et al. 2007; Ćurković Perica 2008a, b). Also, serial dilutions of total DNA (i.e. 20, 10 and 5 ng μL^{-1}) were used as templates in PCR to determine a possible lower titer of phytoplasma in the treated shoots. Direct PCR assays were performed using universal phytoplasma primer pair R16F1/R0 (Lee et al. 1995), for the amplification of highly conserved 16S rDNA. The amplification products were diluted and reamplified in the first nested PCR with primers R16F2n/R2 (Gundersen and Lee 1996). The additional second nested PCRs were performed using R16(I)F1/R1 (LEE et al. 1994) only for the shoots treated with 1 mM of quercetin.

Results

Shoot length and fresh weight increase were used as parameters for measuring the effect of quercetin on '*Ca.* P. asteris'-infected and non-infected *C. roseus* shoots (Tab. 1). None of the used quercetin concentrations showed any beneficialeffect on phytoplasma-infected plants. On the contrary, quercetin (100 μM and 1000 μM) added to the medium resulted in increased severity of symptoms, accompanied by leaf yellowing and browning, and emergence of black spots on leaf edges. Healthy periwinkle shoots treated with quercetin (100 μM) also expressed stunting accompanied by leaf yellowing and slight browning. At the highest concentration of quercetin (1 mM), plants infected with '*Ca.* P. asteris' started to decay shortly after the transfer to the quercetin-supplemented medium, during the first subculture. Shoot elongation and fresh weight increase of both, infected and non-infected plants, treated with quercetin (100 μM or more), were lower than those of untreated controls (Tab. 1), and there was no remission of symptoms in infected plants. After '*Ca.* P. asteris'-infected *C. roseus* shoots were grown through three subcultures on media with different quercetin concentrations, molecular analyses using consecutive PCR reactions with R16F1/R0 and R16F2n/R2 primer pairs showed the presence of '*Ca.* P. asteris' in all of the tested samples on media supplemented with 10 μM or 100 μM quercetin and in positive control (Fig. 1). When total DNA in the concentration of 20 ng μL^{-1} or 10 ng μL^{-1} was used as template, all samples were positive in the direct PCR, but PCR using template

Tab. 1. Effect of different quercetin concentrations on shoot elongation and fresh weight increase on phytoplasma-infected *Catharanthus roseus* shoots in vitro.

	Substance and concentration	Shoot elongation[1]	Fresh weight increase[1]
Phytoplasma infected	Quercetin[2] (10 μM)	1.16±0.13 c	1.22±0.1 c
	Quercetin[2] (100 μM)	1.01±0.07 d	1.04±0.06 d
	Quercetin[2] (1000 μM)	decay	decay
	EtOH (1‰)	1.19±0.11 c	1.33±0.23 c
	control	1.21±0.12 c	1.39±0.27 c
non-infected	Quercetin[2] (100 μM)	1.48±0.2 b	2.48±0.26 b
	EtOH (1‰)	1.82±0.31 a	3.16±0.59 a
	control	1.86±0.45 a	3.2±0.48 a

[1] Mean ± standard deviation. Shoot elongation and fresh weight increase were calculated as the ratio of the final and the initial shoot length or fresh weight, respectively. Means labeled with the identical letters are not significantly different at the 95% level of confidence.

[2] Quercetin stock solution was prepared in ethanol (1‰ final concentration in the medium

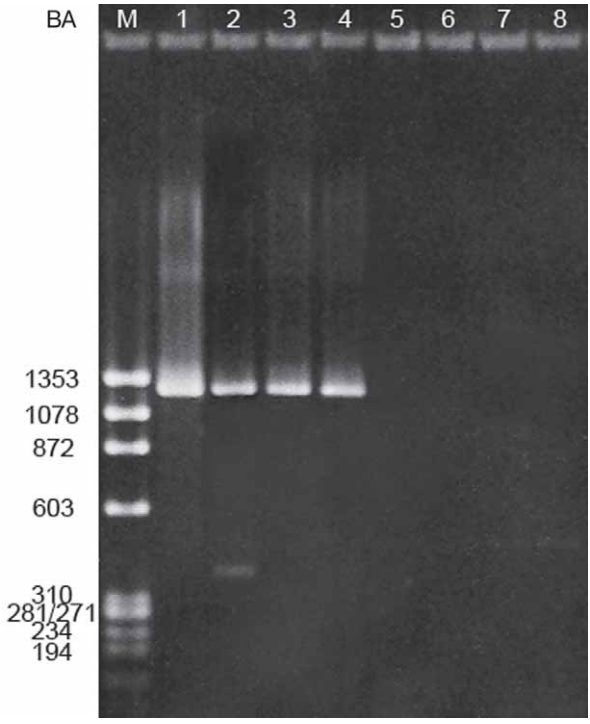

Fig. 1. Agarose gel (1%) electrophoresis of nested PCR amplification products of phytoplasma 16S rDNA obtained using primer pair R16F1/R0. **M** – Molecular weight marker ϕX174 *Bsu*RI digested; **BA** – '*Ca.* P. asteris' –infected *Catharanthus roseus* shoot from the medium supplemented with: 2.2 μM 6-benzylaminopurine. **1** – PCR positive control, **2** – 10 mM quercetin, **3** – 100 mM quercetin, **4** – ethanol; **5** – healthy *C. roseus* shoot from the medium supplemented with: 2,2 μM BA, **6** – 100 mM quercetin, **7** – ethanol, **8** – water control.

in the concentration of 5 ng μL^{-1} DNA did not amplify visible PCR product in samples treated with 10 μM or 100 μM quercetin (Fig. 2). However, phytoplasma presence was again confirmed in all samples in nested PCR (Fig. 3). For the shoots treated with 1 mM quercetin an additional second nested PCR, using the primer pair R16(I)F1/R1, was performed after the first subculture. Phytoplasma was detected in seven out of eight tested samples, although shoots were already decaying. Therefore, the fact that phytoplasma was detected in the second nested PCR or was not detected at all in one shoot is irrelevant since this high quercetin concentration (1 mM) also caused the decay of the host.

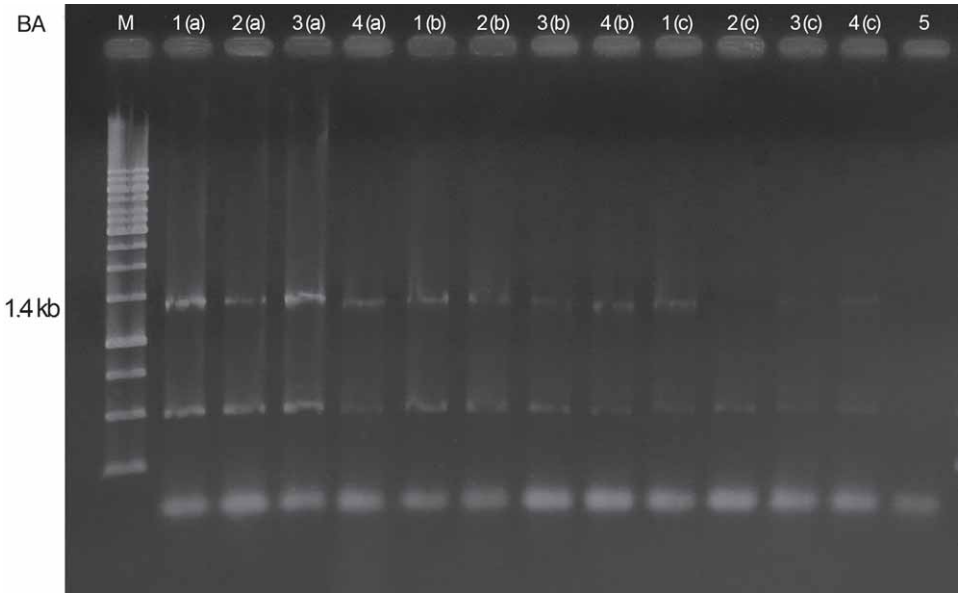

Fig. 2. Agarose gel (1%) electrophoresis of direct PCR amplification products of phytoplasma 16S rDNA obtained using primer pair R16F0/R1. **M** – Molecular weight marker 1kb ladder (Frementas); **BA** – '*Ca.* P. asteris' –infected *Catharanthus roseus* shoot from the medium supplemented with: 2.2 μM 6-benzylaminopurine. **1** – PCR positive control, **2** – 10 mM quercetin, **3** – 100 mM quercetin, **4** – ethanol, **5** – water control. DNA concentration is indicated by the letter **a**=20 ng mL^{-1}, **b**=10 ng mL^{-1}, **c**=5 ng mL^{-1}

Discussion

Reports on quercetin toxicity in plants are quite ambiguous, and while some researchers found no deleterious effects of quercetin in the concentration range of 10–1000 μM on *Arabidopsis* plants (Reigosa and Pazos-Malvido 2007), others found that quercetin in the concentration of 100 μM and 333 μM was toxic to the same species (Parvez et al. 2004). Basile et al. (2000) found out that quercetin inhibits seed germination of *Raphanus sativus*. In our experiments, quercetin supplemented to the medium in the concentration of 100 μM, and higher, caused leaf yellowing and browning in both non-infected and infected periwinkle shoots. The experience from other experimental systems shows that the mode of application can greatly influence the outcome of quercetin treatment (Cushnie and Lamb 2005).

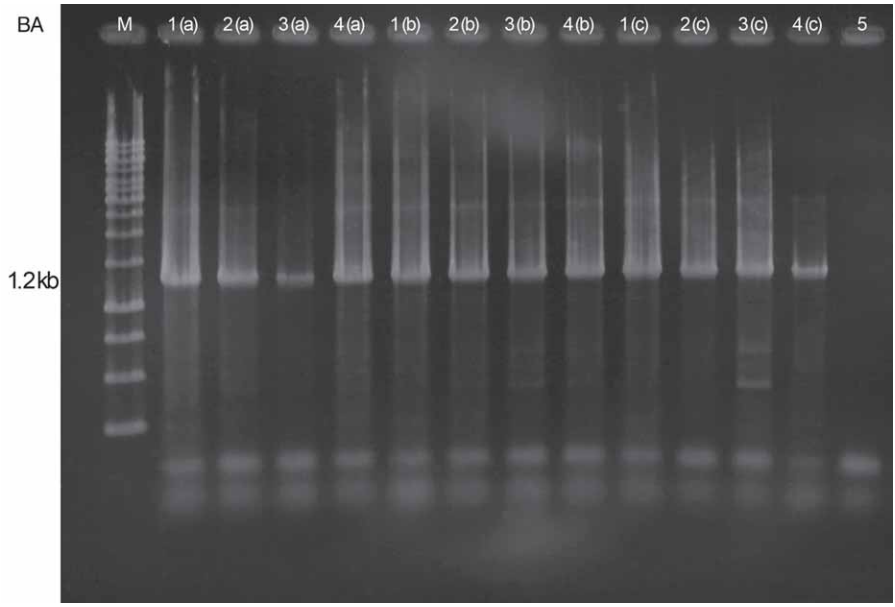

Fig. 3. Agarose gel (1%) electrophoresis of nested PCR amplification products of phytoplasma 16S rDNA obtained using primer pair R16F2/R2. **M** – Molecular weight marker 1kb ladder (Frementas); **BA** – '*Ca.* P. asteris' –infected *Catharanthus roseus* shoot from the medium supplemented with: 2.2 μM 6-benzylaminopurine. **1** – PCR positive control, **2** – 10 mM quercetin, **3** – 100 mM quercetin, **4** – ethanol, **5** – water control. DNA concentration is indicated by the letter **a**=20 ng mL^{-1}, **b**=10 ng mL^{-1}, **c**=5 ng mL^{-1}

Since in our experiment quercetin was supplied to plants via liquid medium, which would allow better uptake of this flavonoid through the plant's vascular system, it is quite plausible that concentrations used in this experiment could have been toxic to healthy and infected plants.

Although quercetin treatment proved to have bacteriostatic or bactericidal effect against many bacteria (CUSHNIE and LAMB 2005), the titer of phytoplasma was only slightly affected in the shoots treated with 10 μM or 100 μM quercetin. Moreover, the severity of the symptoms increased in treated phytoplasma-infected plants. The undesirable effect of quercetin treatment, resulting in the stunting of non-infected and infected plants and in increased severity of symptoms in infected plants, might be explained by several hypothesized mechanisms. First of all, quercetin may interfere with auxin transport through plants by binding to auxin transport membrane protein complexes (MURPHY at al. 2000) thus disturbing the balance of plant growth regulators in healthy and infected shoots. Phytoplasmas also interfere with auxin transport in plants (ALDAGHI et al. 2009). The addition of quercetin might have increased severity of symptoms in phytoplasma-infected shoots by additionally interfering with auxin transport. Periwinkle shoots infected by '*Ca.* P. asteris' exhibit symptoms like proliferations, leaf curling and internode shortening. However, stunting was even more pronounced in quercetin-treated than in non-treated infected plants, moreover leaf browning and appearance of black spots on the leaf edges appeared on quercetin treated plants.

SPENCER et al. (2003) have showed that quercetin, when present in tissue culture media in concentrations higher than 10 μM, actually promoted oxidative damage of cells. In our experiment, healthy and infected plants performed worse on quercetin- than on control medium, with phytoplasma-infected shoots showing greater susceptibility to potentially toxic effects of quercetin.

The results presented in this paper do not reveal the mechanism by which quercetin increased severity of the symptoms in phytoplasma–infected periwinkles, but it is possible that its negative effect is a consequence of several mechanisms. The supplement of quercetin had no curative effect on '*Ca.* P. asteris'. Although quercetin slightly reduced phytoplasma titer in *C. roseus* tissues, its' detrimental effect on plantlets outweighs the possible benefits. The addition of quercetin made symptoms of phytoplasma infection even worse, probably due to synergistic effect of phytoplasma and quercetin as an auxin transport inhibitor.

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