THE EFFECT OF RUTHENIUM RED, A Ca^{++} CHANNEL BLOCKER, ON A RED PIGMENT FORMATION IN MECHANICALLY WOUNDED SCALES OF *Hippeastrum* x *hybr*. hort., AND ON THE GROWTH AND DEVELOPMENT OF *Phoma narcissi*

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A B S T R A C T

Mechanical wounding of *Hippeastrum* bulb scales and other organs induces red colouration. It was found that ruthenium red (RR), a Ca^{++} channel blocker, at concentrations 0.1, 0.2 and 0.5 mM substantially inhibited the red pigment formation in wounded scales of *Hippeastrum*, proportionally to the concentration used. RR, at concentration of 0.3 mM, totally inhibited the mycelium growth of *Phoma narcissi,* a pathogen of *Hippeastrum*, on PDA medium. However, RR did not inhibit development of the disease symptoms caused by *P. narcissi* on scales and basal plate of *Hippeastrum* bulbs. It is suggested that blockage of Ca^{++} influx by ruthenium red inhibits jasmonate biosynthesis and signalling pathway mediating the red pigment biosynthesis and accumulation.

Key words: ruthenium red, *Hippeastrum* x *hybr.* hort., *Phoma narcissi*, red pigment, mechanical wounding, scales, *in vitro*

INTRODUCTION

Calcium functions as a versatile messenger in mediating plant responses to hormones, biotic and abiotic stress signals and a variety of developmental processes (Reddy and Reddy, 2004). Several studies have shown rapid increase of cytoplasmic Ca^{++} concentrations following wounding, but the role and nature of Ca ion fluxes in the wound response is not fully understood (de Bruxelles and Roberts, 2001). Rapid increase of endogenous levels of jasmonates, mainly jasmonic acid (JA), was documented after mechanical wounding of different plant organs, pathogen

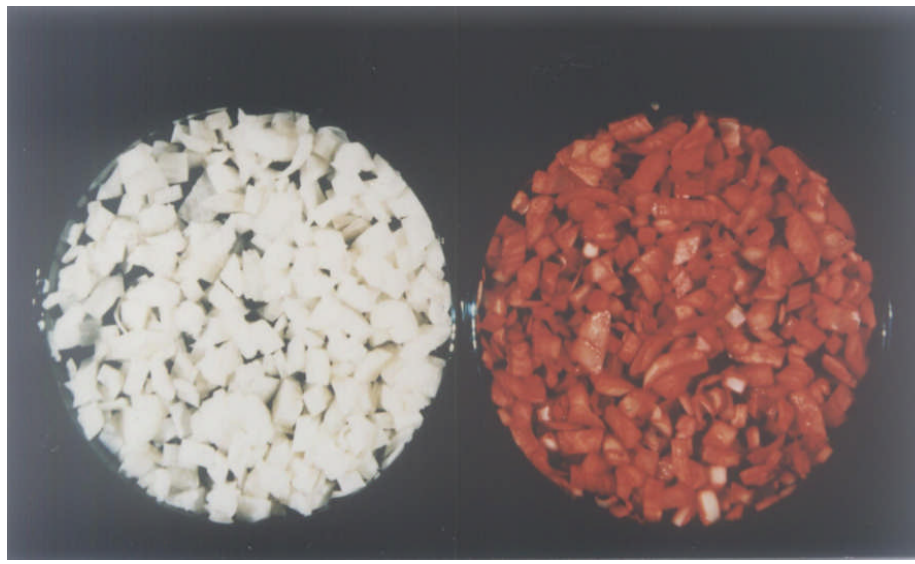

Figure 1. Reddish colouration of mechanicaly injured scales of *Hippeastrum* bulbs: on left – scales immediately after cutting, on right – scales 5 days after cutting

infection or insect attack, as well as under stresses of other kinds (Creelman and Mullet, 1995; Laudert et al., 2000; Mei et al., 2006). Calcium may play multiple roles in wound signaling, including differential regulation of JA-dependent and JA-independent pathways (Blume et al., 2000; Leoń et al., 2001; Sun et al., 2006). Ruthenium red (RR) is the inhibitor of voltage-dependent calcium channels on plasma membrane and cyclic adenosine-5'-diphosphoribose (cADPR) -dependent channels on tonoplast (Muir et al., 1997).

Various organs of *Hippeastrum* infected by *Phoma narcissi*, infested with mite, *Steneotarsonemus laticeps* or mechanically wounded, produce a red pigment on the surface of injured tissues (Fig. 1) (Saniewska, 1998).

The aim of the work was to study the effect of ruthenium red, a known Ca^{++} channel blocker, on the red pigment formation in wounded scales of *Hippeastrum* bulbs, and on the *in vivo* and *in vitro* growth and development of *Phoma narcissi*.

MATERIAL AND METHODS

Hippeastrum x *hybr*. hort. 'Jan', was used in the study. The stock culture of *Phoma narcissi* was maintained on potato-dextrose-agar (PDA) slants at 25°C in the dark. Ruthenium red (RR) was purchased from Sigma-Aldrich Chemicals.

The effect of ruthenium red (RR) on the red pigment accumulation in wounded scales of *Hippeastrum*. The scales were cut into small pieces (dimension ca 4 x 4 mm)

and dipped for 2 hours in either 0.1, 0.2 or 0.5 mM of aqueous solutions of RR, or in water (control). Then the liquids were drained off and tissue samples were kept in closed Petri dishes at 20-25°C in darkness at high humidity. Analyses of red pigment content were carried out after 48, 72, 96 and 120 h of incubation. One gram of the tissue was extracted with 9 ml of 90% methanol. Absorbance of such obtained extract was measured at 495 nm and the results presented in absorbance units.

In another experiment, the effect of 0.5 mM RR, applied to pieces of *Hippeastrum* scales 24 and 48 hrs after cutting, on red pigment level was measured following protocol as above.

The development of *Phoma narcissi* on the scales and basal plate tissues treated with ruthenium red. The bulbs of *Hippeastrum* were vertically cut into halves. After cutting, the basal plate tissues and scales were dipped for 2 hours in either 0.2 and 0.5 mM RR, or in water (control), and then either immediately or after 4-day-delay were inoculated with *P. narcissi.* Disks of mycelium, 5 mm in diameter, were taken from 5-days-old culture of *P. narcissi* grown on PDA. One disk was placed on the surface of basal plate, while on scale surface two disks were placed. The size of necrosis (length and depth) caused by *P. narcissi* on basal plate and scales was measured after 3, 6 and 7 days of incubation. Five bulbs were used for each treatment and the experiment was repeated twice.

***In vitro* growth of *Phoma narcissi* in the presence of ruthenium red.** PDA medium was supplemented with of 0.1, 0.2, 0.3 and 0.5 mM of ruthenium red by adding the compound, dissolved in distilled and sterilized water and additionally filtered through 0.22 µm filter (Millex-GV), to the medium after autoclaving, when temperature dropped to 50°C. Five mm in diameter plugs were taken from 7-day-old culture of *P. narcissi*, and placed in the middle of 90 mm Petri dishes containing PDA medium, supplemented with RR as above. Control plates contained the culture growing on PDA without RR. Five Petri dishes were used as an experimental unit and the trial was repeated twice. After 6 days of incubation in darkness at 25°C, diameter of fungal colonies was measured in two perpendicular directions.

The data of all experiments were subjected to an analysis of variance and Duncan's multiple range tests at 5% of significance was used for means separation.

RESULTS AND DISCUSSION

Mechanical wounding of *Hippeastrum* bulbs scales induced their red colouration (Fig. 1). Ruthenium red (RR) at concentrations 0.1, 0.2 and 0.5 mM substantially inhibited red pigment formation in wounded scales of *Hippeastrum*, proportionally to the concentration used (Fig. 2). Treatment with RR 24 and 48 hrs after wounding inhibited red pigment formation to a much lesser extent than treatment executed directly after wounding (Fig. 3).

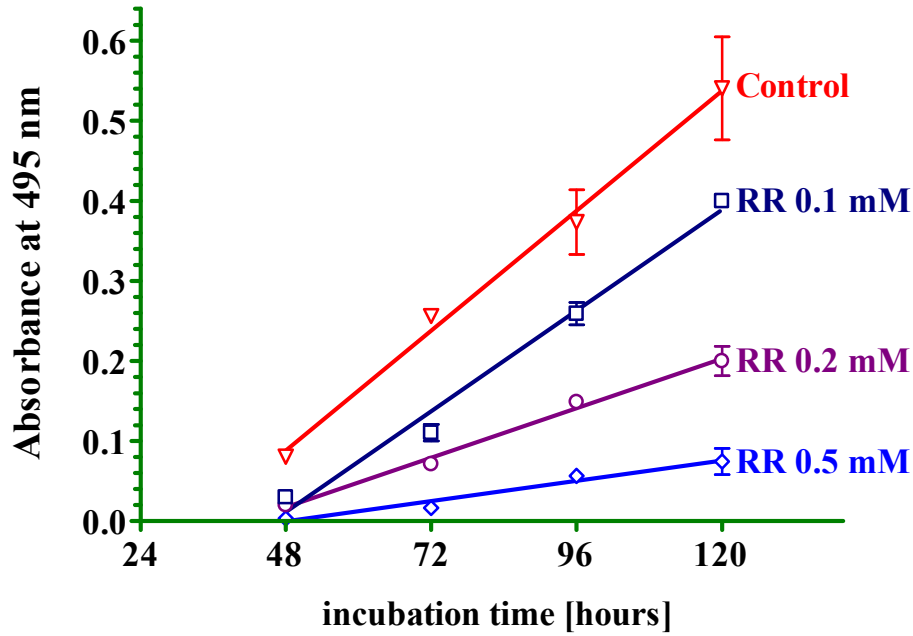

Figure 2. The effect of ruthenium red (RR) on red pigment content in wounded scales of *Hippeastrum* bulbs; RR was applied directly after wounding. The vertical bars represent standard deviation

It is well known that triggering the action potential by heat treatment or mechanical wounding stimulates jasmonic acid biosynthesis and PINII gene expression in higher plants (Peña-Cortés et al., 1995). Pretreatment of plants with ruthenium red, a known Ca^{++} channel blocker, completely inhibited the increase in cytosolic calcium content during membrane potential depolarization that was associated with heat-stimulated action potential and inhibited induction of jasmonic acid biosynthesis and PINII gene expression (Fisahn et al., 2004). Plant defense systems against biotic (pathogen and insects invasion) and abiotic (mechanical wounding) stresses can be divided into two major categories: synthesis of secondary metabolites and specific proteins. It is well known that jasmonates represent an integral part of the signal transduction chain between stress signal(s) and stress responses.

Thus, it is suggested that Ca^{++} influx is required for biosynthesis of secondary metabolites as defense factors (phytoalexins), probably through the jasmonate pathway signaling; blockage of Ca^{++} influx by ruthenium red inhibits jasmonate biosynthesis and signaling pathway mediating the red pigment accumulation in wounded scales of *Hippeastrum*. It should be mentioned that Ca^{++}, applied as 100 mM calcium chloride, caused a substantial increase of red pigment

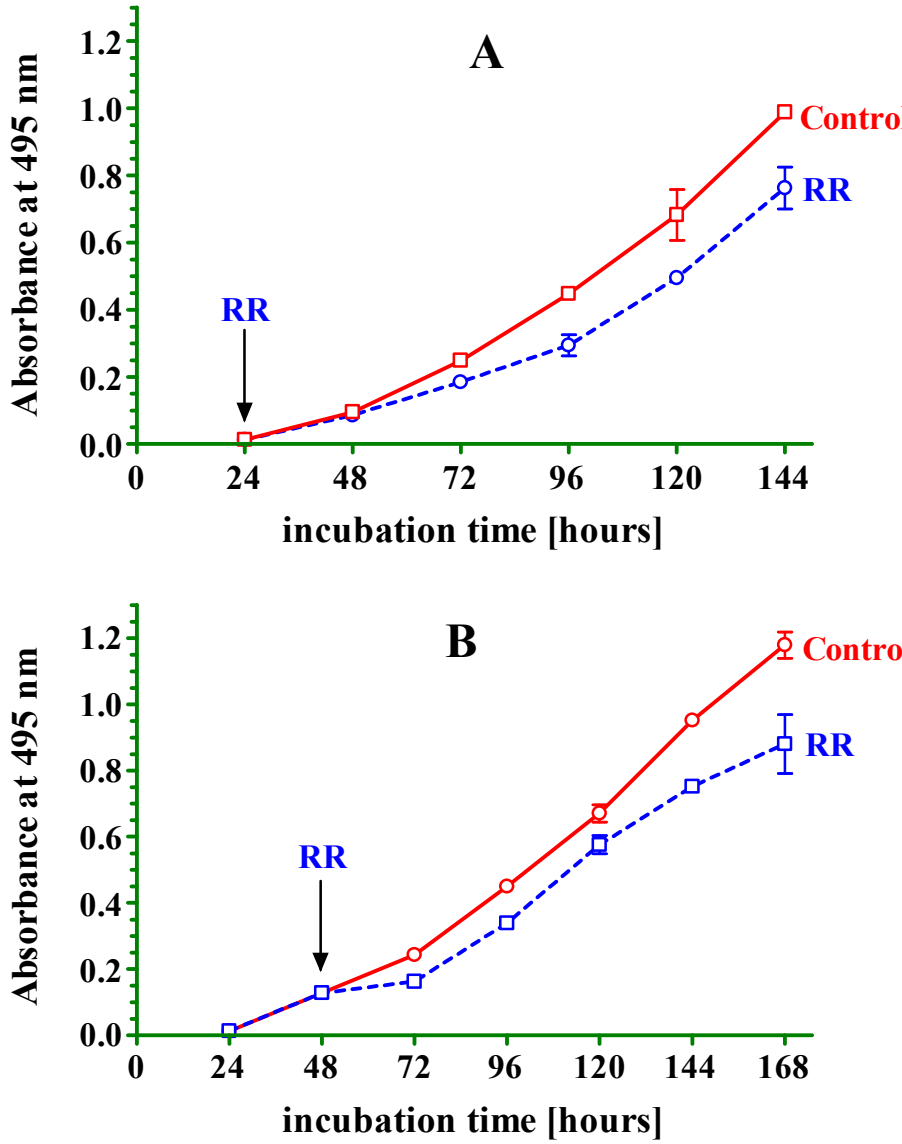

Figure 3. The effect of ruthenium red (RR) on red pigment content in wounded scales of *Hippeastrum* bulbs; RR (0.5 mM) was applied 24 h (A) and 48 h (B) after wounding. The vertical bars represent standard deviation

formation in wounded *Hippeastrum* scales, whereas EDTA, which complexes Ca^{++}, inhibited the pigment accumulation (Wink and Lehmann, 1996).

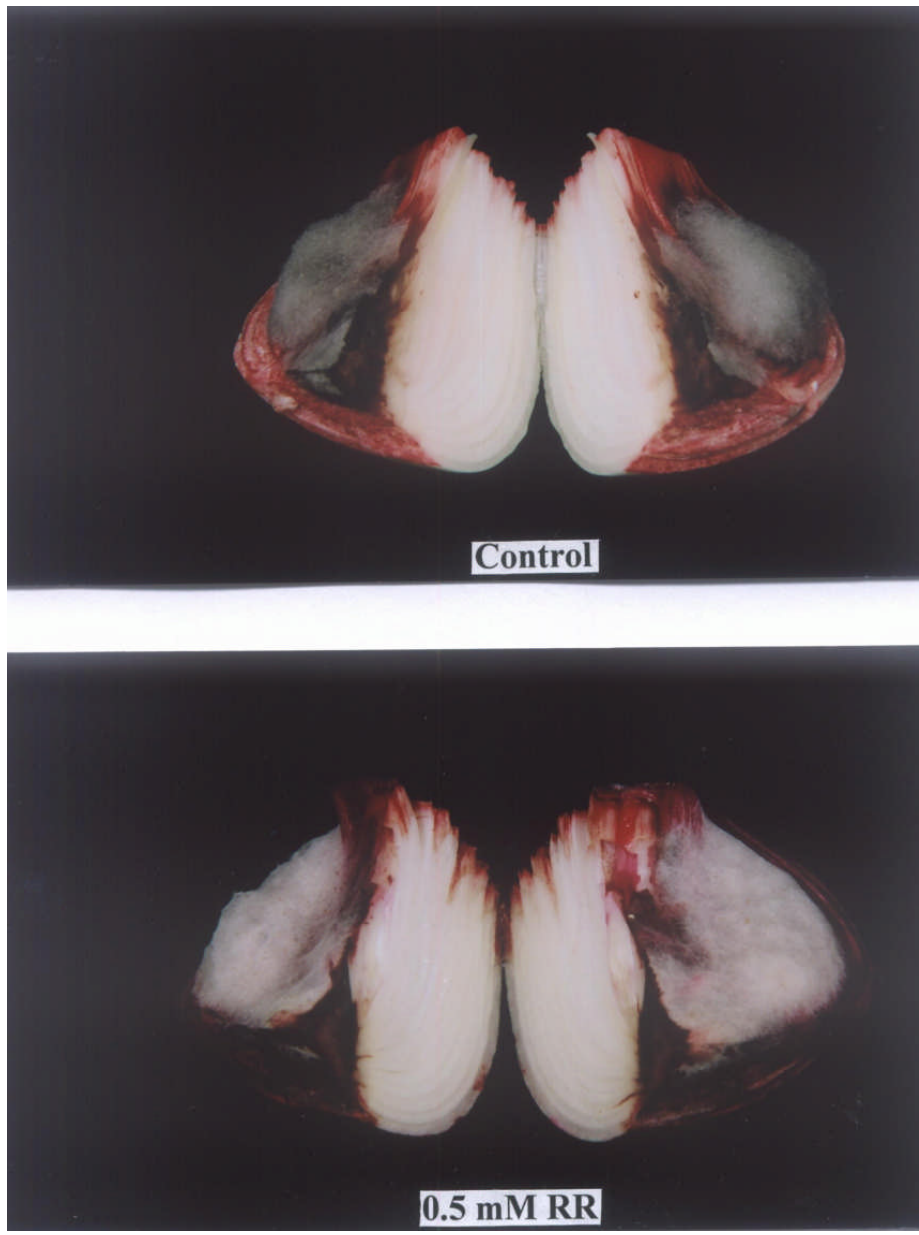

Figure 4. The effect of ruthenium red (RR) on development of *Phoma narcissi* disease in scales of *Hippeastrum* bulbs; scales were inoculated immediately after treatment with RR

Table 1. The effect of ruthenium red (RR) on development of *Phoma narcissi* on basal plate and scales of *Hippeastrum* bulbs; plant tissues were inoculated immediately after treatment with RR

Treatment	Length of necrosis [mm] after days				Depth of necrosis [mm] 7 days after inoculation	
	3		6			
	basal plate	scales	basal plate	scales	basal plate	scales
Control	15.0 a*	20.0 b	20.0 a	27.5 a	10.0 a	14.2 b
0.2 mM RR	12.0 a	16.6 a	20.0 a	30.0 a	7.5 a	7.3 a
0.5 mM RR	12.7 a	15.0 a	21.2 a	27.5 a	9.0 a	10.0 a

*Means in columns followed by the same letters are not significantly different at $P \leq 0.05$

When inoculation of cut halves of *Hippeastrum* bulbs with *Phoma narcissi* took place directly after treatment with RR, the development of disease was not affected, as compared to the control (Fig. 4, Tab. 1). When the inoculation was done after mechanical injuries, when red pigment appeared on scales and basal plate of *Hippeastrum*, the fungus *Phoma narcissi* practically did not induce disease symptoms. However, treatment of injured organs with RR, which greatly inhibited accumulation of the red pigment (Fig. 2), restored growth of the pathogen in a rate comparable to the control (Fig. 5, Tab. 2). It is possible that the red compound formed in wounded tissues or in tissues infected by *P. narcissi*, which is of a flavan nature (Wink and Lehmann, 1996; Saniewska and Budzianowski, 1997; Budzianowski and Saniewska, unpublished), is bound to cell wall and in this way plays a defensive role against fungal infection by blocking the cell wall degradation enzymes produced by the pathogen.

Ruthenium red at concentration 0.3 mM totally inhibited the mycelium growth of *P. narcissi* on potato-dextrose-agar (PDA) medium (Fig. 6, 7). Thus, it seems that Ca^{++} plays also an important, but unknown yet role in mycelium growth of *Phoma narcissi* and probably other fungal pathogens as well. It was found earlier that the growth rate and morphology of hyphae of various fungi are affected by factors which influence also intercellular Ca^{++} (Jackson and Heath, 1993; Levina et al., 1995).

CONCLUSION

These observations suggest that Ca^{++} influx during wounding of *Hippeastrum* bulbs may mediate elicitor signal to the jasmonate pathway and then lead to the production of red pigment; blocking of Ca^{++} influx by ruthenium red inhibits jasmonate biosynthesis and signaling pathway mediating the red pigment biosynthesis and accumulation.

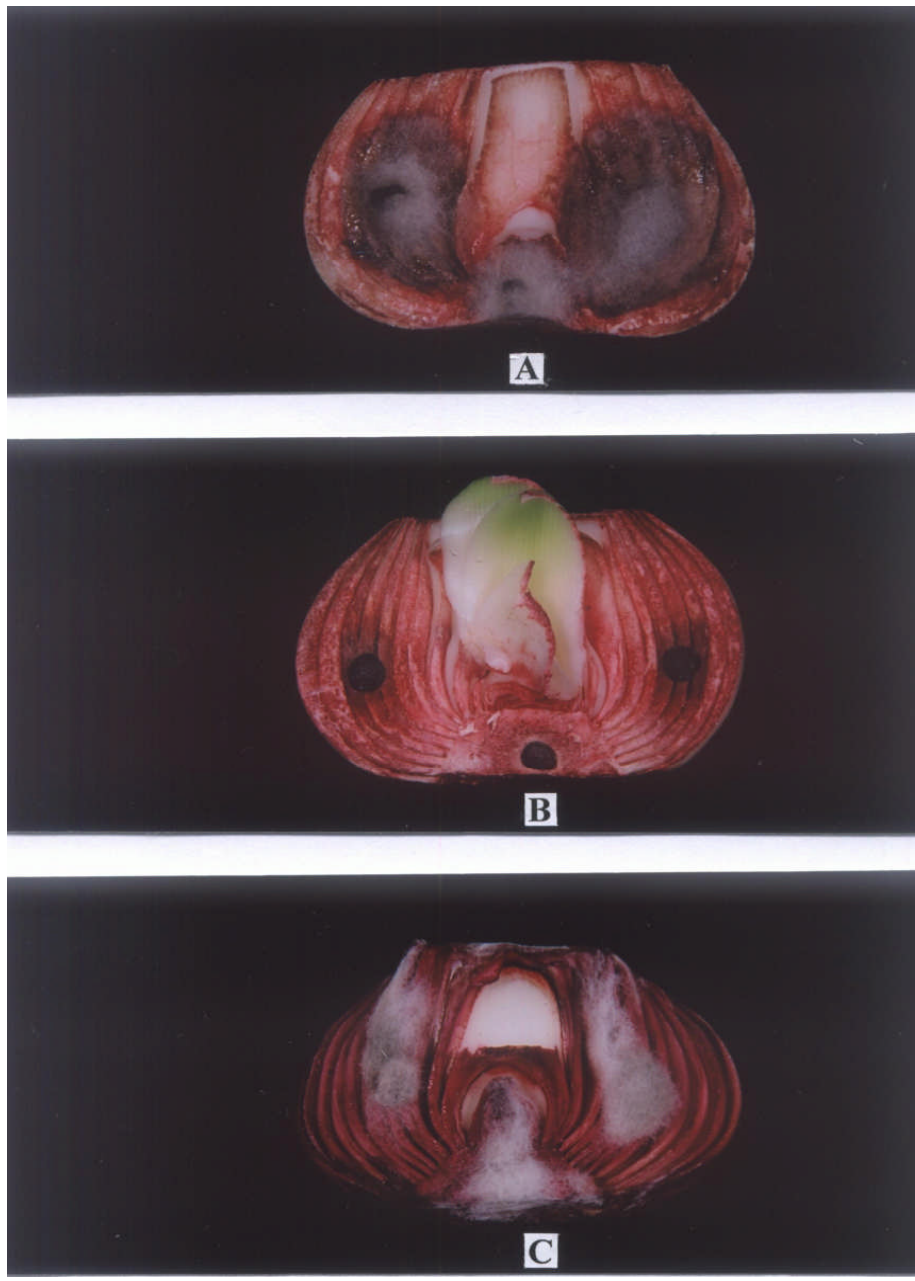

Figure 5. The effect of ruthenium red (RR) on development of *Phoma narcissi* disease in basal plate and scales of *Hippeastrum* bulbs; A – inoculation carried out immediately after cutting, B – inoculation carried out 4 days after cutting, C – inoculation carried out 4 days after cutting and treatment with 0.5 mm RR

Table 2. The effect of ruthenium red (RR) on development of *Phoma narcissi* on basal plate and scales of *Hippeastrum* bulbs; plant tissues were inoculated immediately and 4 days after cutting

Treatment	Length of necrosis [mm] after days				Depth of necrosis [mm] 7 days after inoculation	
	3		6			
	basal plate	scales	basal plate	scales	basal plate	scales
Inoculation carried out immediately after cutting:	10.7 c*	20.0 d	13.7 b	24.5 d	10.0 c	12.7 c
Inoculation carried out 4 days after cutting:						
Control	1.6 a	6.5 a	3.5 a	11.5 a	2.5 a	3.5 a
0.2 mM RR	7.0 b	10.0 b	13.7 b	14.5 b	8.5 b	8.5 b
0.5 mM RR	5.7 b	11.2 b	12.5 b	20.8 c	7.5 b	10.0 b

*Explanations, see Table 1

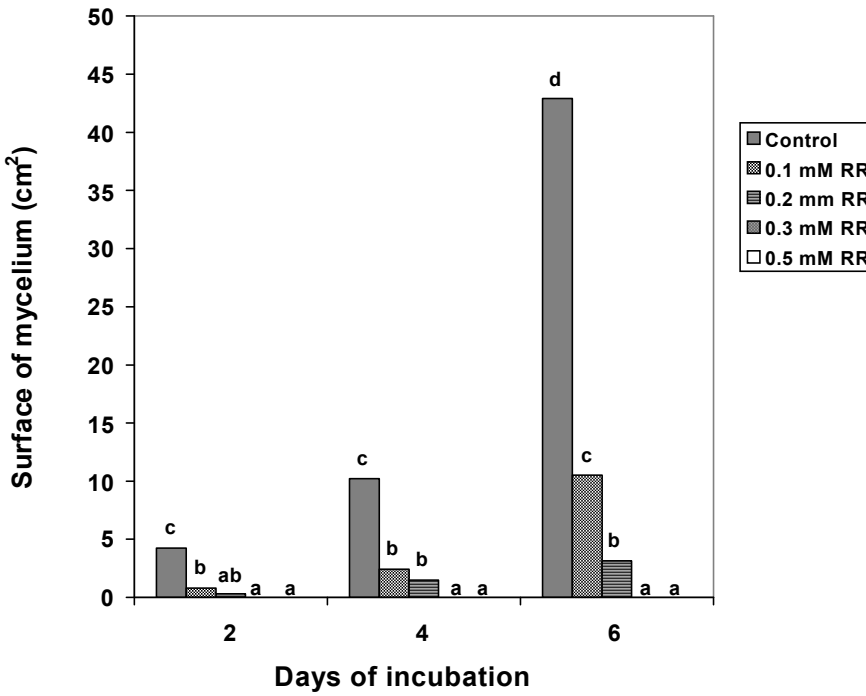

Figure 6. The effect of ruthenium red (RR) on the mycelium growth of *Phoma narcissi* on PDA medium

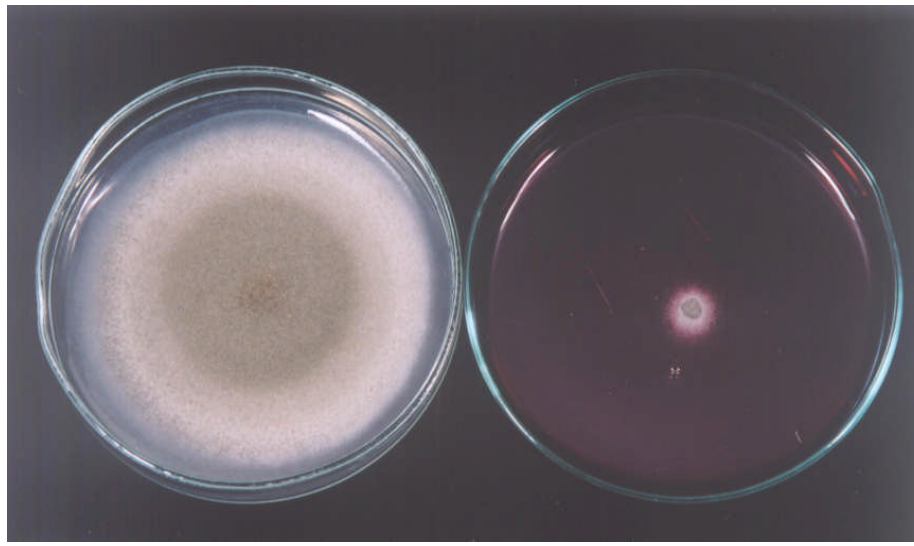

Figure 7. The effect of ruthenium red (RR) on the mycelium growth of *Phoma narcissi* on PDA medium; on left – control, on right – 0.2 mM RR

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WPŁYW CZERWIENI RUTENOWEJ, INHIBITORA KANAŁÓW WAPNIOWYCH, NA TWORZENIE SIĘ CZERWONEGO BARWNIKA W MECHANICZNIE USZKODZONYCH ŁUSKACH *Hippeastrum* x *hybr.* hort. I NA WZROST I ROZWÓJ *Phoma narcissi*

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S T R E S Z C Z E N I E

Mechaniczne uszkodzenie łusek cebul lub innych organów *Hippeastrum* indukuje tworzenie się czerwonego barwnika. Wykazano, że czerwień rutenowa (RR), znany inhibitor kanałów wapniowych, w stężeniu 0,1, 0,2 i 0,5 mM silnie hamuje tworzenie się czerwonego barwnika w uszkodzonych łuskach cebul *Hippeastrum*, proporcjonalnie do zastosowanego stężenia. RR w stężeniu 0,3 mM całkowicie hamuje *in vitro* wzrost grzybni *Phoma narcissi*, patogena *Hippeastrum*, na pożywce PDA. Jednakże, RR nie hamuje rozwoju choroby powodowanej przez *P. narcissi* na łuskach i piętce cebul *Hippeastrum*. Sugeruje się, że zahamowanie wypływu Ca^{++} do cytoplazmy przez czerwień rutenową hamuje biosyntezę jasmonianów i sygnalny mechanizm pośredniczący w biosyntezie i akumulacji czerwonego barwnika w uszkodzonych łuskach *Hippeastrum*.

Słowa kluczowe: czerwień rutenowa, *Hippeastrum* x *hybr*. hort., *Phoma narcissi*, czerwony barwnik, uszkodzenie mechaniczne, łuski, *in vitro*