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BIOLOGICAL ACTIVITY OF *PHYTOPHTHORA CRYPTOGEA* CULTURE FILTRATE

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Abstract

Studies are carried out about the effect of the culture filtrate of *Phytophthora cryptogea* on different plant species and animal smooth muscle samples.

The results point out that in the 16 plant species, treated with the culture filtrate of *P. cryptogea*, different types of response are registered: systemic, expressed by yellowing of the veins, leading to a sudden wilting of the plants; necrotic (local), shown as quick chlorosis and necrosis of the plant cells; local-systemic types – a systemic infection in the veins next to the necrotic spots.

On the smooth muscles, the effect of the filtrate has a two-phase profile. In concentrations up to 1.5 %, the filtrate stimulates the muscle tonus. In a higher concentration (≥ 2 %) the filtrate has an inhibitory effect. This effect is shown in two cases: when calcium ions (Ca^{2+}) in an extracellular solution are reduced, and when the Ca^{2+}-influx through the membrane is blocked.

The biphasic effect supports the following propositions: (1) the low concentration of the culture filtrate depolarizes the membrane, increases the Ca^{2+}-influx and stimulates the smooth muscles; (2) the higher concentration of the culture filtrate hyperpolarizes the membrane, decreases the Ca^{2+}-influx and induces the relaxation of the smooth muscles.

Keywords: *Phytophthora*, culture filtrate, defense response in plants, smooth muscles, isometric contraction.

INTRODUCTION

Phytophthora species are between the most specific plant pathogens based on their biological, physiological characteristics and the response to exogenic influences during the pathogenicity process, unknown within other genera in the Kingdom of Fungi (Gregory, 1983; Zentmyer, 1983; Griffith et al., 1992; Erwin and Ribeiro, 1996). The complicated "pathogen-plant" interaction in the genera *Phytophthora* has not been fully understood yet.

Calcium ions (Ca^{2+}) are considered to be extremely important as a secondary messenger in the regulation of the metabolic processes connected with the development of the plant responses to the different exogenic impact (Ermakova et al., 2005; Goodman & Novacky, 1994). Within plants, the Ca^{2+} are distributed among a great number of cell structures as it is distributed in animals (Rasmussen and Barrett, 1984; Stab & Ebel, 1987; Marme, 1989; Goodman & Novacky, 1994).

These ions influence the permeability and structural stability of the plasmalemma, as well as the intermolecular bonds of the proteins in the cell wall membrane, responsible for the cellular stability (Ermakova et al., 2005). The Ca^{2+} play an important role in the pathological process and the defense reactions, such as resistance and hypersensitivity (Goodman & Novacky, 1994).

Irrespective of the Ca^{2+} essential role in the membrane transport and signaling, their role in the development of the hypersensitive reaction is not quite clear. Atkinson et al. (1990) report that Ca^{2+}-influx in tobacco cells is stimulated during the first few hours of the hypersensitive reaction (combined with K^{+}-efflux through the plasma membrane leading to extracellular alkalinization).

This increased Ca^{2+}-influx was prevented by calcium channel blockers. They assume that the Ca^{2+}-influx through the cell membrane or the tonoplast can be influenced by the condition of the Ca^{2+}-channels or Ca^{2+}-APTases (Hille, 1984; Briskin, 1990).

Atkinson et al. (1990) also state that Ca^{2+} plays a role in the hypersensitive response induction through different signaling pathways similar to those in the animals (Rasmussen & Barrett, 1984; Marme, 1989; McAinsh & Pittman, 2009).

The plant systems can use Ca^{2+}-dependant phospholipases in signal pathways similar to the animals' ones. The fact that La^{3+} suppress both the phytoalexin induction (Stab & Ebel, 1987) and the hypersensitive reaction (Atkinson et al., 1990), supports the statement that Ca^{2+} may play the leading role in the plant defense.

Apostol et al. (1989) and Ermakova et al. (2005) think that the Ca^{2+}-influx might accompany the first oxidative burst of the cell. They assume that the damage to the cell plasma membrane of active oxygen forms is responsible for the Ca^{2+}-influx.

The ability of some *Phytophthora* species to induce the hypersensitive reaction through the release of elicitins or elicitors, and to stimulate phytoalexin synthesis is also studied (Stössel, 1988; Keen, 1990; Keen & Yoshikawa, 1990; Hardham & Blackman, 2010).

Elicitor molecules show different specific levels and induce different responses such as susceptibility and resistance. They are not specific (Darvill & Allersheim, 1984; Dixon, 1986; Lamb et al., 1989). *P. parasitica* and *P. cryptogea* purified elicitor causes local and distal hypersensitive reaction in *Nicotiana* sp., radishes and turnips, but not in 12 other species included in the experiment (Kamoun et al., 1993; 1994).

In the literature, there are not enough data about the reaction of different plant species to the culture filtrate from the pathogen *P. cryptogea*. The biological activity of the same culture filtrate on the animals' smooth muscle samples, as well as the influence of this filtrate on some elements of Ca^{2+} homeostasis, is not studied also.

MATERIALS AND METHODS

Plant pathogen

Phytophthora cryptogea is a plant pathogen from genus *Phytophthora*, class *Oomycetes*, kingdom *Stramenopila*. The *Oomycete* is isolated from apple trees (Bjaga village, Pazardzhik region) showing symptoms of *Phytophthora* root and stem rot. Isolation is done on selective PARP medium. Identification of the pathogens from the genus *Phytophthora* is carried out based on morphological characteristics and molecular methods (Erwin and Ribeiro, 1996).

The pathogen is grown on Pea liquid media, 250 ml flasks, at 25^0C, in a dark growth chamber. After 10 – 12 days the cultural liquid is filtrated through sterile microbiology membrane filter (Pall Corporation). The cultural filtrate is divided into 10 ml samples, in sterile tubes and stored in a fridge, at 2^0C.

Plant materials

Plant materials used in the tests, belong to the following plant species: apple (*Malus*) seedlings, stage 2-4th leaves; almond (*Prunus amygdalus*) – seedlings, stage 2-4th leaves; peach (*Persica*) – seedlings, stage 2-4th leaves; plump (*Prunus*) – seedlings, stage 2-4th leaves; strawberry (*Fragaria*) – rooted plants, stage rosette and leaf inoculation; pear (*Pyrus*) – leaf inoculation; quince (*Cydonia*) – leaf inoculation; peach (*Persica*) – seedlings, stage 2-4th leaves and leaf inoculation; black currant (*Ribes*) – leaf inoculation; linden (*Tilia*) – leaf inoculation; vines (*Vitis*) – leaf and shoot inoculations; rose (*Rosa*) – leaf inoculation; spindle tree (*Evonymus europeus*) – leaf and leaf stalks inoculation; geranium (*Geranium*) – leaf inoculation; peony (*Paeonia*) – leaf and leaf stalks inoculation; lilac (*Siringa*) – leaf inoculation; lettuce (*Lactuca*) – leaf and leaf stalks treatment.

In vivo test with different plant species

In vivo tests are carried out with the culture filtrate of *P. cryptogea*, in a growth chamber (Vindon Scientific, UK), at 26^0C and relative humidity 80 %, at 12 hours of light to 12 hours of darkness.

The variants include:

- Plant species are dipped into the culture filtrate of *P. cryptogea* without any dilution or 1:1 dilution, for 24 hours.
- Drops of the culture filtrate are injected into the leaf petiole or into the veins by a sterile syringe.

The leaves and the roots are disinfected with pure alcohol, prior treatment. The observations of the developed symptoms are carried out daily, in order to register the appearance of the first symptoms: systemic, necrotic (local) and local-systemic.

In vitro physiological test

Isometric force measurements (Jespersen et al., 2015) are performed on the smooth muscle samples (length 15.0 ± 1.5 mm).

The preparations are taken from a rat stomach, separated in a circulatory direction, without mucosa, and are placed in an isolated tissue bath system with modified Krebs solution (in mmol/l): 120 NaCl, 5.9 KCl, 15.4 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.5 glucose.

The solution is aerated (with 95 % O_2 and 5% CO_2) and temperature-controlled (36 ± 1) ^{0}C to create optimal conditions for the manifestation of the spontaneous contractile activity. One gram of the preload is applied to the tissues. The equilibrium period is 1 hour.

The isomeric tension is investigated in the presence of 0.5; 1.0; 1.5 and 2% of the *Phytophthora* culture filtrate.

The evoked contractile activity is triggered with 50 mmol/l KCl. Methoxyverapamil (D600, Knoll AG, Germany) and Tetraethylammonium chloride (TEA, Sigma-Aldrich) are used as a blocker of the voltage-operated calcium channels ($VOCa^{2+}C$) and the Ca^{2+}-sensitive K^+ channels (BK family of K^+ channels) respectively.

The registration of the isometric tension is performed using the strain-gauge-measuring bridge (Swema, Sweden) and multichannel polygraph (Linseis, Germany). All data are expressed as a percentage of maximal 50 mmol/l KCl contractions.

The results are presented as (mean ± SEM) values of at least five independent measurements.

RESULTS

The effect of the cultural filtrate of *Phytophthora cryptogea* on the development of the symptoms in plants.

In response to the treatment with *Phytophthora cryptogea* culture filtrate, 16 plant species develop the following basic types of symptom reactions – systemic, necrotic or local-systemic (Table 1).

Table 1. Symptoms response of 16 plant species treated with culture filtrate of *P. cryptogea* (*in vivo*)

No.	Plant species	Symptoms development in plants			
		dipped in culture filtrate		injected with culture filtrate	
		No dilution	Dilution 1:1	Into the veins	Into the parenchyma
1	Apples *(Malus)*	Systemic infection	Systemic infection	Systemic infection	Necrotic spots
2	Pear (*Pyrus*)	–	–	Systemic infection	Necrotic spots (1 – 3 mm)
3	Quince (*Cydonia*)	–	–	Systemic infection	Necrotic spots (0.5 – 1 mm)
4	Peach (*Persica*)	Systemic infection	Systemic infection	Systemic infection	Necrotic spots (3 – 4 mm), with fine chlorotic halo (0.5 mm)
5	Almond (*Amygdalus*)	Systemic infection	Systemic infection	Systemic infection	Necrotic spots (3 – 4 mm), and vein clearing
6	Plump (*Prunus*)	Systemic infection	Systemic infection	Systemic infection	Necrotic spots (1 – 3 mm), and vein clearing
7	Black currant (*Ribes*)	–	–	Systemic infection	Necrotic spots (0.5 – 2 mm) with fine halo, and vein clearing
8	Linden (*Tilia*)	–	–	Systemic infection	Necrotic spots (1 – 4 mm)
9	Vines (*Vitis*)	–	–	Vein necrosis on leaves(1 – 2 mm), necrotic spots on shoots	Necrotic spots (2 – 4 mm)
10	Strawberry (*Fragaria*)	Systemic infection	Systemic infection	Systemic infection and vein necrosis	Necrotic spots and vein clearing
11	Rose (*Rosa*)	–	–	Systemic infection and necrotic lines	Necrotic spots
12	Spindle tree (*Evonymus europeus*)	–	–	Systemic infection	Necrotic spots
13	Geranium (*Geranium*)	–	–	Systemic infection, with necrotic lines	Necrotic spots (2 – 3 mm)
14	Peony *(Paeonia)*	–	–	Systemic infection, with necrotic lines	Necrotic spots (2 – 3 mm)
15	Lilac (*Siringa*)	–	–	Systemic infection, with fine necrotic lines	Necrotic spots (0.5 – 2 mm) with chlorotic halo (0.1 – 0.2 mm)
16	Lettuce (*Lactuca*)	–	–	Peripheral vein clearing, with necrotic lines	Necrotic spots (0.5 – 2 mm)

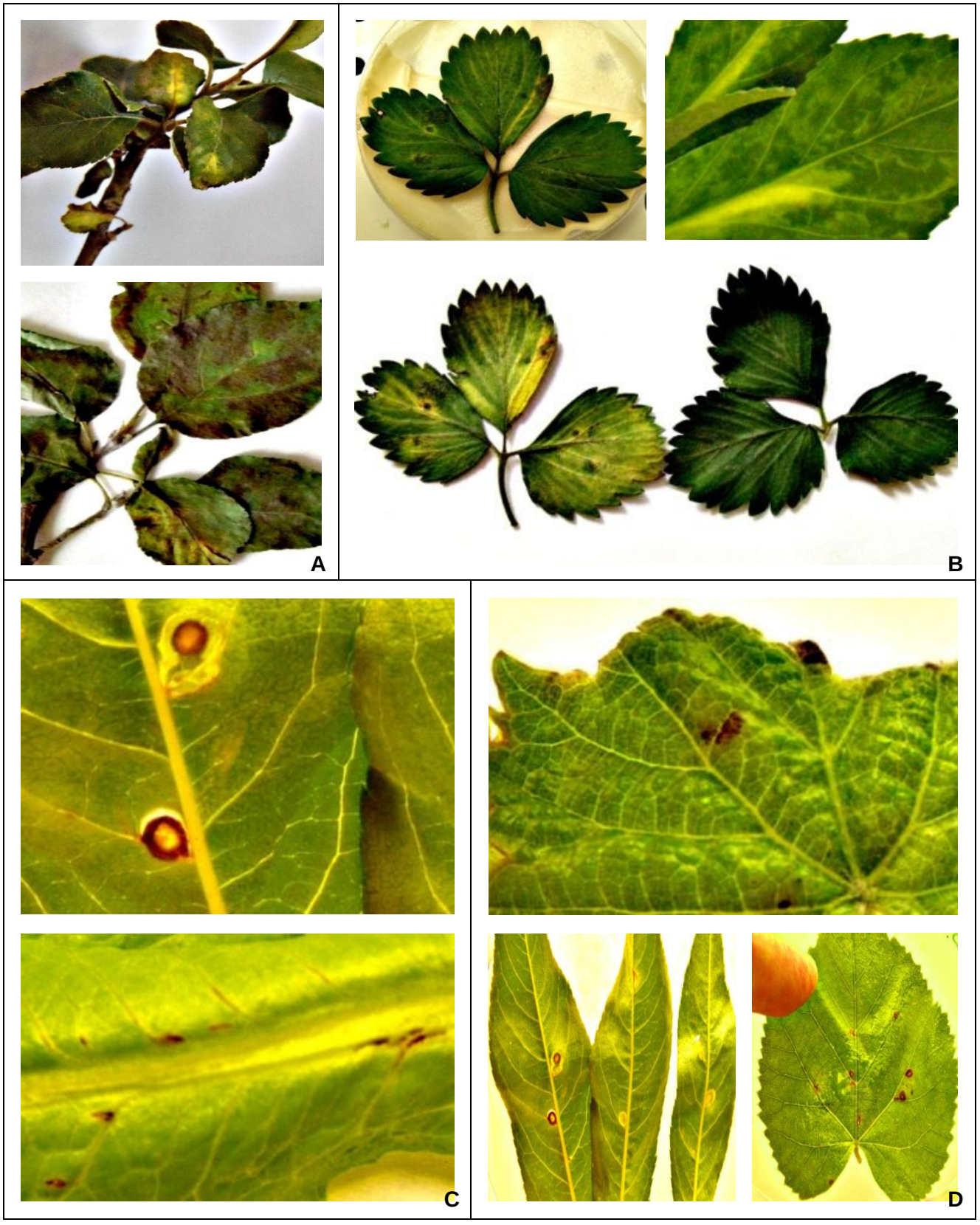

Fig. 1. *Symptom reactions of different plants to culture filtrate of Phytophthora cryptogea*

A systemic type of infection develops when plant roots are dipped into culture filtrate or when it is injected into the leaf veins (Figure 1). The symptoms include: vein yellowing, followed by sudden wilting of the plant tops (Figure 1 A, B). They appear first 24 to 48 hrs after the treatment.

A necrotic type of response develops when the filtrate is injected into the parenchyma cells after 3-4 days. It is expressed in a quick plant tissue chlorosis, followed by a rapid tissue necrosis and a cell collapse, or an appearance of a halo around the necrotic tissue (Figure 1C).

In some plant species, the response to the filtrate is also expressed with the systemic infection in the veins close to the necrotic spots – a local-systemic type of symptoms (Figure 1D).

The effect of the culture filtrate of *Phytophthora cryptogea* on rat's smooth muscle samples

• a dose-response effect

The dose-response curve of the *Phytophthora cryptogea* culture filtrate is illustrated in Figure 2. The filtrate brought into the bath solution in an increasing concentration – from 0.5 to 2%, has a biphasic effect. With the increasing of the concentration up to 1.5%, the culture filtrate increases the smooth muscle isometric tension. In a concentration higher than 1.5%, the effect changes into an inhibitory one on the muscle tone. The effect of the culture filtrate is reduced in the case of a pre-depolarized membrane, experimentally achieved by 5 mmol/l KCl, applying to the extracellular media.

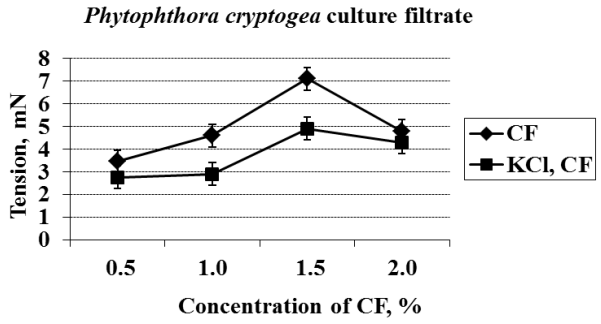

Fig. 2. *Dose-response curve of Phytophthora culture filtrate (CF). The isometric tension of the smooth muscle samples is without (CF in the legend) and with (KCl, CF in the legend) introduction of KCl in the bath solution. Average values of tension are presented as mean ± SEM (n = 5 separated samples)*

• a Ca^{2+} reduction and a voltage-operated Ca^{2+}-channels ($VOCa^{2+}C$) blocking.

When the Ca^{2+} are reduced in the extracellular media, the effect of the 2% culture filtrate is slightly reduced compared to the control value (Figure 3). A similar effect is observed when the membrane calcium channels $VOCa^{2+}C$ are initially blocked (prior treatment with D600 10^{-6} mol/l). The culture filtrate induced contraction, after KCl-membrane depolarization, is reduced in comparison with the control isometric tension.

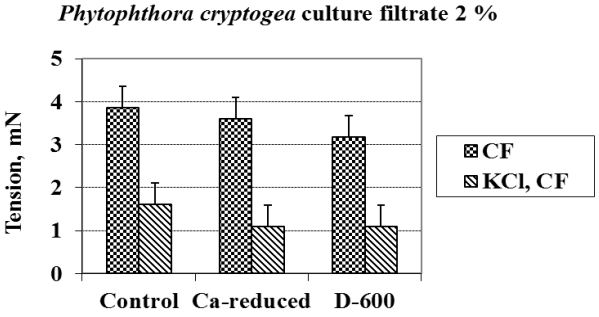

Fig. 3. *The effect of Phytophthora culture filtrate in the Ca^{2+}-reduced solution and after the Ca^{2+}-influx blocking with D-600. The control is the tension in the normal (extracellular Ca^{2+}-composition) bath solution. Average tension values are presented as mean ± SEM (n = 7 separated samples)*

• a blocking of the K^+-efflux

The effect of the culture filtrate after blocking the K^+-efflux through the Ca^{2+}-sensitive K^+channels (BK channels) with TEA is illustrated in Figure 4.

When the K^+-efflux is blocked in advance, the effect of a 2% culture filtrate is reduced. The decrease of the muscle tonus tension is better manifested at a lower concentration of the blocking agent – 10^{-6} mmol/l TEA.

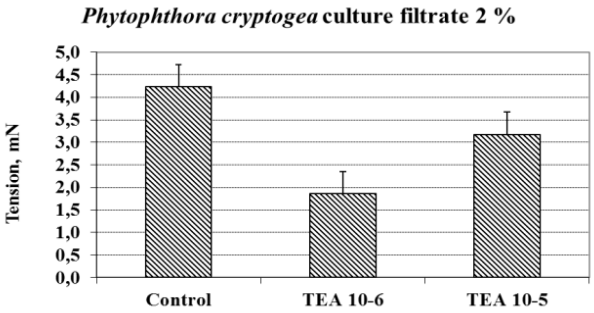

Fig. 4. *The influence of 10^{-6} and 10^{-5} mmol/l TEA on the effect of Phytophthora culture filtrate 2%. Average tension values are presented as mean ± SEM (n = 5 separated samples). The control is the isometric tension without TEA in the extracellular solution*

DISCUSSION

In response to the pathogenic attack, the plants have developed defense mechanisms. One of the most efficient mechanisms is the hypersensitive response. That response can be triggered by the metabolic products elicited from some pathogens (Kamoun S. et al., 1993; 1994; Bonnet P., et al., 1996; Blein J-P et al., 2002).

The 16 plant species treated with the culture filtrate of *Phytophthora cryptogea* show the following basic types of reactions to the inoculation (Table 1):

- a systemic infection: veins yellowing, plant tops sudden wilting and tissue cell collapse;
- a necrotic type, expressed in three different ways: (1) a quick plant tissue chlorosis and necrosis, a tissue cell collapse; (2) a halo around the necrotic tissue; (3) an infection progress along the veins near the necrotic lesions;
- a local-systemic type: systemic infection in the veins close to the necrotic spots.

In some plant species from the Families *Cruciferae* (radishes and turnip) and *Solanaceae* (tobacco) the elicitins, secreted from the different *Phytophthora* species, induce the hypersensitive response locally or at some distance from the inoculation point. Mezzetti et al. (1994) state that the culture filtrate of *P. cactorum*, activates the plasma membranes in the cells of the apple rootstocks sensitive to the pathogen but does not affect the cells of the resistant ones.

The proteins exuded by *P. quercina* and *P. gonapodyides* in culture media cause the chlorosis and the necrosis of the tobacco leaves while those from *P. citricola* show weak symptoms (Heiser et al., 1999).

Our results support the statement that *Phytophthora cryptogea* elicits biologically active substances in the culture filtrate. These substances cause different responses in the plants. Darville & Allershein (1984), Dixon (1986), Lamb et al. (1989) state that the culture filtrate of *Phytophthora cryptogea* is not specific, but is a factor of the pathogenic process similar to the phytoalexins. This filtrate can activate the initial plant resistance signals.

In response to different stimuli, the concentration of Ca^{2+} within the plant cell cytoplasm increases being a signal for transferring information (Stab & Ebel, 1987; Goodman & Novacky, 1994; Ermakova et al., 2005). There are lots of similarities in the variation of free Ca^{2+} in the cytosolic system within plants and animals (Siebers et al., 1990; Mahadi & Beecher, 1994; Messiaen & Van Custem, 1994; Buch, 1995; Ishihara et al., 1996). The researchers point out that the elicitors can influence the activity of the plasma membrane and the Ca^{2+}-channels (Zimmermann et al., 1977; Gelli et al., 1997). The Ca^{2+} increase the resistance to the microbe enzymes by stabilizing the cell membrane.

The present experiment about the influence of the culture filtrate on the smooth muscle samples (taken from the rat's stomach) allows the discussion of the role of Ca^{2+} in the pathogenicity process and the appearance of the hypersensitive response in the plants (Goodman & Novacky, 1994; Nemchinov, 2008).

The *Phytophthora* culture filtrate has a stimulating effect on the smooth muscle samples. This effect is proportionally dependent on the culture filtrate concentration in doses from 0.5 to 1.5% in the extracellular solution (Fig. 2). At 2% the effect on the spontaneous contractile activity is reduced. This biphase profile of the dose-effect curve of the culture filtrate supports the statement about:

- The low culture filtrate concentration depolarizes the membrane and increases the Ca^{2+}-influx. It leads to the stimulation of the smooth muscle;
- The high culture filtrate concentration hyperpolarizes the membrane due to activating of the K^+-efflux through the Ca^{2+}-sensitive K^+channels (through the membrane depolarization and the increased intracellular Ca^{2+} in synergistic manner) and decreases the Ca^{2+}-influx. It leads to the relaxation of the smooth muscle (Cui 2005; Berridge 2014). This effect is reduced if the membrane is pre-depolarized with KCl (Fig. 2).

Figure 3 illustrates the dependence of the inhibitory phase of the culture filtrate on the amount of the intracellular Ca^{2+}. The later can change due to the Ca^{2+}-reduced solution (the reduced concentration of extracellular Ca^{2+}) or when the entry of Ca^{2+} is blocked by a prior treatment with D-600.

The blocking of the high conductivity Ca^{2+}-sensitive K^+ channels (i.e., BK-channels) with TEA (Fig. 4) stimulates the inhibitory effect of the culture filtrate on the smooth muscle samples tonus when the concentration of the blocking agent is low (10^{-6} mmol/l), and K^+-efflux is not highly inhibited.

The literature data point out that the pathogen during its early biotrophic stages secretes substances that inhibit or activate the plant defense systems (Darville & Allersheim, 1984; Dixon, 1986; Lamb et al., 1989; Jiang et al., 2006). The increased K^+-efflux from the susceptible plant cells leads to the increasing of the K^+ around the pathogen. This causes the pathogen membrane depolarization, the improved Ca^{2+}-influx through the membrane and the pathogen multiplication (Goodman & Novacky, 1994). This assumption is supported as quoted in the literature:

- the culture filtrate of *P. cactorum* changes the trans-membrane electrical potential only in the susceptible apple rootstocks (Mezzetti et al., 1994);
- Trifluoperazine (the blocker of the Ca^{2+}-binding protein calmodulin) has a suppressive effect on the sporangia formation in *Phytophthora palmivora* and on the gametangia formation in *Phytophthora cantorum* (Elliott, 1986; 1988).

The possible reason for this increased K^+-efflux might be the formation of the active oxygen forms damaging the plant membrane and leading to Ca^{2+}-influx (Heinstein, 1986; Atkinson et al., 1990; Goodman & Novacky, 1994), because of the high Ca^{2+}-gradient (the difference in the Ca^{2+} concentrations outside:inside is 20000:1).

The sudden increase of the intracellular Ca^{2+} induces the K^+-efflux through Ca^{2+}-sensitive K^+-channels at a low (SK) and high conductivity (BK) (Atkinson et al., 1990). This K^+-efflux leads to the hyperpolarization of the plant membrane and overcomes the defense mechanisms due to the decrease Ca^{2+}-influx.

CONCLUSIONS

The data analysis indicates that *Phytophthora cryptogea* elicits biologically active substances in the culture filtrate. These substances are biologically active on the plants and cause different responses in different species: systemic, necrotic or local-systemic. They also affect the smooth muscle samples.

Analyzing the results about the influence of the culture filtrate of *P. cryptogea* on the smooth muscle samples we can suggest that at the initial stages of the pathogen development, a low concentration of its metabolites (in the culture filtrate) depolarizes the membrane of the cells, changes the potential of the cell membrane and improves the Ca^{2+}-influx. With a higher concentration of the metabolites, the cell membrane depolarization and the increased intracellular Ca^{2+}-concentration lead to K^+-efflux through Ca^{2+}-sensitive K^+-channels. That could be a switch-on mechanism for the expression of the defense reaction in the plants also.

Based on the experiments carried out we can support the hypothesis (Goodman and Novacky 1994; Ermakova et al. 2005) about the specific role of the Ca^{2+} in the hypersensitive reaction unlocked by the *P. cryptogea* culture filtrate.

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