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EFFECT OF CRUDE CULTURE FILTRATES OF THE PATHOGENIC FUNGUS PHOMA MEDICAGINIS ON *IN VITRO* CULTURES OF PEA

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Abstract

Biotic stress is one of the major causes for considerable yield loses and limits in plant performance. To cope with this problem, multidisciplinary approach is applied and research is carried out at several levels of organization of the living organisms. The large scale studies are due to the fact that resistance is a complex of genetic, physiological, biochemical and other mechanisms. It includes plant-pathogen interactions demonstrated on organism, cellular and molecular level. Biotechnology affords an opportunity for application of alternative methods to investigate stress response and to select for higher tolerance. Essential prerequisites for this kind of work are the in vitro culture system and a stress factor which is applicable in vitro and simulates the natural stress factor on cellular or tissue level. In this respect culture filtrates from pathogenic fungus can be used as a selective factor in plant cell in vitro cultures. The objectives of the study were to define the appropriate culture filtrates from a pathogenic fungus which can be used for in vitro modeling of biotic stress using plant tissue cultures. Long-term organogenic pea cultures and crude culture filtrates from the virulent isolate of the pathogenic fungus Phoma medicaginis var. Pinodella causing ascochytosis disease were used. The negative effects of crude culture filtrates obtained at different stages of fungus growth were studied recording changes in pea bud and shoot induction and development. The virulence of the crude culture filtrate was tested after being subjected to cold or hot sterilization. The culture filtrate after the 5th day of fungus suspension initiation demonstrated suppression of the pea organogenesis. The negative effect is strongest on the 9th day of fungus cultivation. The cold sterilization of the fungus filtrate by Millipore filter with 0.2 μ membrane pores is the most effective and reliable. However, it is more difficult compared to autoclaving which is reliable, too, but decreases culture filtrate activity.

Key words**:** *biotic stress, in vitro modeling, pea, Pisum sativum, Phoma medicaginis, ascochytosis*

INTRODUCTION

Pea is the most commonly produced grain legume in Europe and second-most in the world [11]. Ascochyta blight is a serious disease of cool-season grain legumes (pea, lentils, faba bean and chickpea) [10]; Ascochytosis is widespread throughout the major pea-growing areas, especially in temperate regions in Europe [3]. Severe epidemics may result in total crop failure [2]. Seed treatment and foliar application of fungicides, as well as cultural practices are often unsuccessful and uneconomical to control the disease. The use of cultivars with high levels of resistance is considered the most economical solution for long-term disease management. However, breeding for resistance has been challenged by several factors, including the limited sources of good resistance [8].

Biotic stress causes considerable crops yield loses and limitation of plant performance. Overcoming this problem is not an easy task due to the various mechanisms and to the great number of genes involved in stress resistance. The research large scale is due to the fact that resistance is a complex of genetic, physiological, biochemical and other mechanisms, what demands multidisciplinary approach in investigating and overcoming sensibility to unfavourable conditions.

Research is on organism, cell and molecular levels. The characterization of genotypes that may contain different genes for resistance may help breeders to develop better and more durable resistance, compared to the current one. Genetic relationships of germplasm accessions differing in reaction to ascochyta blight could be evaluated using Simple Sequence Repeats (SSR) markers linked to Quantitative Trait Loci (QTL) for resistance [8]. PCR techniques based on amplification of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions have been used widely for the differentiation and detection of closely related fungal species [9].

Lack of natural gene resources is an additional limitation in screening for higher tolerance and development of new resistant varieties. This imposes the application of different approaches in selection of genotypes and varieties with the desired traits. In this respect, biotechnological agriculture aims at avoidance of growth reduction due to a specific pathogen, permitting a wider range of crop species and varieties to give higher yields under wider range of conditions [1]. "Green" plant biotechnology allows application of new methods to study stress response and to select for higher resistance. Plant cell and tissue cultures can be used to create *in vitro* models in order to imitate biotic and abiotic stress. This contributes to facilitating breeding process and to overcome some of the problems of classical breeding. Basic requirements for application of biotechnology in studying stress effects, creating models, tests and schemes for selection are (i) the availability of *in vitro* culture system and (ii) an effective selective agent representing the desired trait on cell or tissue level, (iii) to find the criteria for drawing a parallel between response in vitro on cell/tissue level and in vivo on the level of organism.

The *in vitro* modelling to study the disease resistance is based on the hypothesis that the bacterial and fungi toxins and other biologically active components play a substantial role in host-pathogen interactions, and that a parallel can be drawn between the response on cell level and that one of plant level. When cultured *in vitro* in a liquid medium in flasks, the pathogenic fungus *Phoma medicaginis var. pinodella* (causing ascochytosis) releases into the toxic medium other bioactive substances involved in the disease initiation and development. These crude culture filtrates could be used to simulate biotic stress and to study the biochemical interactions between the host and the pathogens.

Ascochyta blight of pea is caused by three related fungal species, commonly referred to as the Ascochyta complex: *Ascochyta pisi, Ascochyta pinodes, Phoma medicaginis var. pinodella.* The pathogens are often host specific [7]. Ascochyta blight pathogens are all necrotrophic, killing plant cells in advance of mycellial development. Therefore, toxins and cell-wall degrading enzymes are often presumed to be important biochemical determinants of pathogenesis [10].

The objectives of the present investigation were to study the effect of crude culture filtrates on *in vitro* development of organogenic pea cultures taking in consideration the culture period of the fungus and the method for sterilization of the crude culture filtrate.

MATERIALS AND METHODS

In the present investigations, virulent isolates Pmp 8-1 from the pathogenic fungus *Ph. medicaginis var. pinodella* causing ascochytosis disease were obtained from naturally infected pea. The isolates were maintained on potato-dextrose medium solidified with agar-agar at temperature of 20^{o}C in dark. Crude culture filtrates were obtained when three disks of 8 mm in diameter from the developing fungus were transferred into 100 ml potato-dextrose broth in flasks of 300 ml volume. Cultures were agitated on a shaker machine (150 rpm) at 20^{o} C for 7-10 days. During its development in the liquid culture, the fungus formed globules which reached 3-4 cm in diameter on the 10th day. These globules were removed by passing the liquid cultures through cloth and sieves with different pore size (250μ, 125μ, 64μ, 30μ, 10μ). Two methods were applied for the sterilization of the sieved suspension: (i) CF was passed consecutively through sterile Millipore filters with pore size of 1.5μ, 0.8μ и 0.2μ for the elimination of fungus spores, mycelium and other fungus particles; (ii) CF was autoclaved at

120° C at pressure of one atmosphere for 20 min, like the nutrition media. These procedures aimed at obtaining of sterile crude culture filtrate (CCF), which added to the medium for cultivation of plant tissues, will not cause development of fungus infection.

Sterile crude culture filtrate was added into the nutrient medium to reach final concentration of 30 % v/v and 50 % v/v. This was achieved when double strength medium was prepared. When equal volumes of the plant growth medium and the crude culture filtrate were mixed, concentration of the filtrate was 50 %. The other concentration of 30 % was obtained when 3 portions of the filtrate were mixed with 3 portions of distilled autoclaved water and 5 portions of the double strength plant growth medium was added.

Thus prepared, plant growth media containing fungus culture filtrate were used for cultivation of organogenic pea cultures. The latter were initiated from seedling cotyledonary nodes. After the induction of buds and shoot formation, organogenic cultures (Fig.1A, 1B) were maintained and multiplied using our protocols [4, 5, 6].

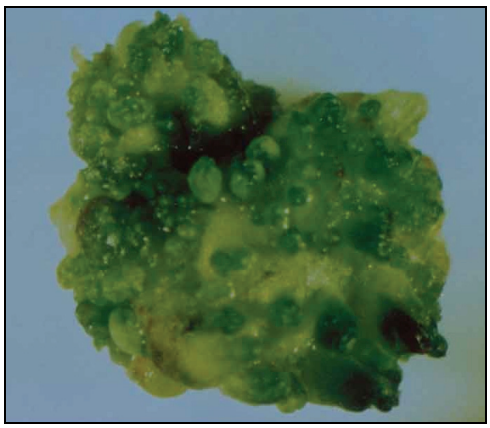


Fig.1A. Organogenic pea culture with numerous buds.

Small clusters of buds with fresh weight of 40 mg were plated on shoot growth OCHMG medium (containing MS macro-salts, 4 folds increased MS micro-salts, Gamborg's B-5 vitamins, 30 g/l sucrose, 7 g/l agar, 5 mg/l benziladenin, 1 mg/l naphtyl acetic acid and 2 mg/l giberrelic acid [6]) with fungus culture filtrate (30 % or 50 % v/v) or without CCF (control). All the cultures were incubated in a cultivation room at temperature of 22°C, photoperiod 16/8 h light. Development and growth of cultures under biotic stress were recorded after one month using following criteria: fresh weight, viability, necrosis, number of green buds.

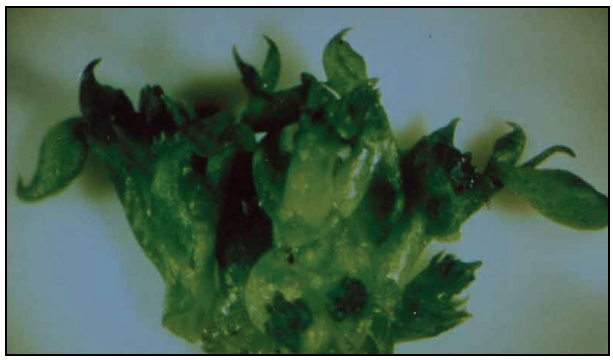


Fig. 1B. Shoot development of pea *in vitro* cultures

RESULTS AND DISCUSSIONS

The sterility test aimed at determination of the optimal conditions for sterilization of the fungus crude culture filtrate. The results in Table 1 indicate that passing the crude culture filtrate through sterile medicinal cloth which retains the fungus globule and followed by sieving through big diameter pores (60μ, 45 μ and even 1.5 μ) does not bring to sterility as fungus spores and mycelium particles remained in the suspension. After being mixed with the cell culture medium, the latter caused its contamination without inoculation of any plant tissue. Sterile CCF was obtained after a series of consecutive passages through sieves with decreasing pores diameter (250 μ, 125 μ, 60 μ, 45 μ). The most reliable sterility was possible when 0.4 and 0.2 μ Millipore was used. However, these filters were used for sterilization of chemical solutions. This indicated that there were some bioactive substances remaining in the CCF which are still pathogenic.

Another observation was that possibility for infection to grow with the aging of the fungus cultures. Spores were released from the mycelium globules into the liquid medium after the 8-9th day of cultivation. Then infection was more severe. Hence, it was recommendable to use crude culture filtrate before the 8th day. At that time, the suspension was more transparent and with lower viscosity and could be easily cleaned from the debris.

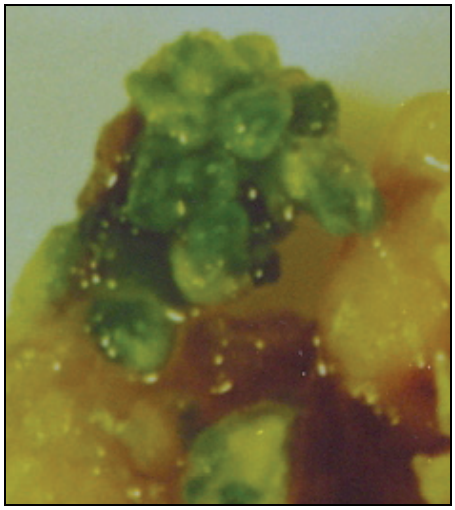
Table 1. Test for elimination of particles from the pathogenic fungus for obtaining sterile cultural filtrate.

First step of elimination of the fungus particles	Second step of elimination of the fungus particles	Contamination
Sterile medicinal cloth	Sieve with pores of 60 μ	yes
Sterile medicinal cloth	Sieve with pores 45 μ	yes
Sterile medicinal cloth	Millipore filter 1.5 μ	yes
Sieve with pores 250 μ, 125 μ, 60 μ, 45 μ	Millipore filter 1.5 μ	no
Sieve with pores 250 μ, 125 μ, 60 μ, 45 μ	Millipore filter 0.4 μ	no
Sieve with pores μ, 125 μ, 60 μ, 45 μ	Millipore filter 0.2 μ	no

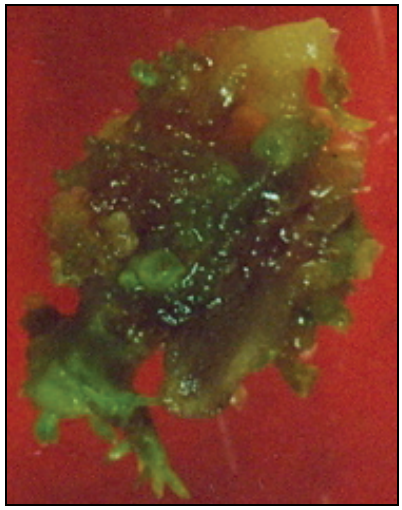
Autoclaving is the most common methods for sterilization of the plant nutritive media. However, in cases when the culture media contains thermo labile components, cold sterilization is applied. In this case, filters Millipore were usually used as they were able to capture bacteria. In our studies, cold sterilization was preferred. However, it was more difficult and time consuming as the filters became chocked very quickly, even after the preliminary sieving of the CCF. Autoclaving was the easier way though the activity of the filtrate dropped down by 30-40 % (Table 2). In our experiments, we further used the method of the cold sterilization. However, autoclaving is also acceptable in the cases when a larger amount of pea genotypes must be screened for disease tolerance. In the latter case, the reduction of the suppressive ability should be taken in consideration.

Table 2. The effect of the different types of sterilization of the fungus cultural filtrate on its suppressive efficiency on *in vitro* development of pea organogenic cultures (Fig. 2)

No Variant	Plant culture medium	Type of sterilization of CCF	Plant tissue Fresh weight [mg]	Growth relative to variant one [%]	Growth relative to variant two [%]	Reduction of the suppressive activity
1	OCHMG (Control)		240.4	100		
2	OCHMG + 50 % CCF	Millipore filter 0.2 μ	56.96	23.56 %	100 %	
3	OCHMG + 50 % CCF	Millipore filter 0.2 μ + autoclaving	98.39	40.93 %	173 %	By 42 %
4	OCHMG + 50 % CCF	Sieves + autoclaving	77.99	32.44 %	138 %	By 30 %



A.



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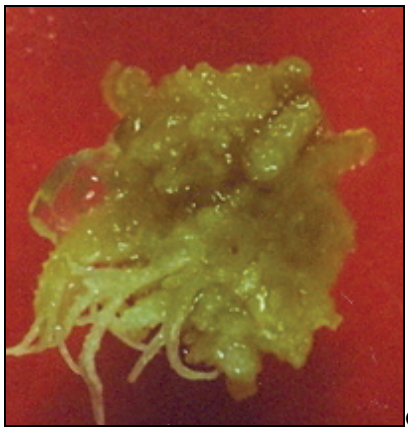 C

Fig. 2. Suppression of organogenesis and bud formation on media containing fungus culture filtrate of: A -30 %, B - 50 %, C - 70 % v/v.

In another series of experiments, we studied the negative effect of the fungus filtrates obtained after various periods of cultivation of the fungus after inoculation of the fungus disks into the liquid medium and different growth of the fungus mycelium (globules) (Table 3).

Table 3. Effect of the period for fungus growth of the efficiency of the fungus culture filtrate to suppress plant tissues growth *in vitro*

No Variant	Period for fungus cultivation [days]	Diameter/Size of fungus globules [cm]	Concentration of the CCF in the plant nutrient media [%]	Multiplication index* of the organogenic cultures buds	Multiplication index relative to the control [%]
1	-	-	0	3.29	100.0
2	5	2-3	30	2.38	72.9
3	5	1-1.5	30	1.86	56.9
4	7	2-3	30	2.25	73.5
5	7	1-1.5	30	2.19	71.6
6	9	4-5	30	1.52	46.6
7	9	2-3	30	1.28	38.8

*Multiplication index represents the ratio between the number of buds in the cluster at the end of the cultivation period to the number of initial buds in the cluster transferred to the medium for growth and multiplication.

The results indicated that CCF was active even on the 5th day of cultivation. A tendency for higher suppression of the CCF was obtained from a suspension containing bigger number of globules which were smaller in size (variant 3 and 7, Table 3). CCF taken on the 9th day of culture expressed the highest suppressive effect on the pea bud/shoot growth. In the variants 6 and 7, induction of new buds was restricted and the index of multiplication was more than two folds lower. As mentioned before, fungus spore formation started on the 8th day and their removal might create problems. That is why the best CCF was obtained on the 7th 8th day when CCF the negative effect was the best.

CONCLUSIONS

The crude culture filtrate after the 5th day of initiation of the suspension culture demonstrates suppression on the development of the pea organogenic cultures, though the biggest negative effect was recorded on the 9th day. It is highly recommendable to use CCF before formation of fungus spores in the liquid medium. Aterilization of the CCF is most reliable when Millipore membranes with pores of 0.2 μ (at the last step) are used. Autoclaved CCF is also active, though in reduced power. In this case, the decrease of the effectiveness should be taken in consideration. The results obtained thus were used for developing a test for tolerance to ascochyta blight and for *in vitro* selection.

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