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# The Use of Interactions in Dual Cultures *in vitro* to Evaluate the Pathogenicity of Fungi and Susceptibility of Host Plant Genotypes

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Additional information is available at the end of the chapter

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## 1. Introduction

The subject of biological and biochemical bases for immune and defense reactions of an organism to e.g. pathogens is a very broadly defined question. Although the substance of immunology has common ground, it is otherwise perceived in the case of humans and animals, while different aspects are highlighted in the case of plant organisms. While in the case of human the emphasis is put on research aimed to develop a variety of therapies to heal autoimmune disorders, in plants the studies focus on the effect of various biotic, abiotic and anthropogenic stress factors on plant organisms. Biotic stress factors include: fungal infectious diseases, insect pests or excessive occurrence of herbivorous mammals while abiotic stress factors include: atmospheric conditions (extreme weather events, relative humidity deficiencies), soil properties e.g. fertility, physiographic conditions or different stress conditions: oxidative, i.e. load of oxygen, sodium chloride, water deficit or stress caused by the effect of heavy metals (lead - Pb, mercury - Hg, iron - Fe etc.), while anthropogenic stress factors include: air, water and soil pollution, forest fires or improper forest management. The impact of anthropogenic factors, which has escalated in recent decades, causes changes in the natural environment and ecosystems of certain regions of the globe. Forest trees, as important elements of the ecosystem, are vulnerable to climate and environmental changes. This suggests that it is important to thoroughly understand and explain the problem of dieback of trees as a result of the impact of stress factors on the forest environment, especially that the determination of the cause-effect relationship is more complex than previously believed.

The phenomenon of dieback of economically important forest trees due to pathogenic fungi as biotic environmental components is not fully understood yet. This can be explained by the fact that the disease process of trees depends among others on the properties of host plants as well as on characteristics of fungi – the potential causal agents of diseases. These both groups of organisms are affected by environmental factors, that may increase or decrease the susceptibility to diseases. In most cases the assessment of the host plant susceptibility and the properties of pathogenic fungi are done by evaluating the frequency of disease symptoms' outbreak in relation to the presence of a certain fungal species and based on the pathogenicity tests. The implementation of such tests is often very difficult, because in the case of forest ecosystems we are dealing with perennial plants.

A new method of research, that shows the relationship (interactions) between the two organisms involving the stimulation or inhibition of their growth was developed in the eighties. This is dual cultures research (involving two organisms), in which one of the organisms is callus (*in vitro* cultured plant tissue that covers plant wounds in embryonic or non-embryonic stadium), while the second organism is the studied fungal species. [4] from the Slovak Academy of Sciences, and [8] from the University of New Brunswick in Canada were the first ones who studied the pathogenicity in dual *in vitro* cultures at the embryonic level. Such research was conducted later in Finland [31, 21], Germany [28, 24], Norway [13, 12], Slovakia [7, 33] and in Great Britain [3].

Based on previous research conducted in Poland [Nawrot–Chorabik unpublished, 19, 20] it may be stated that such experiments are promising, because the development of reliable tests in dual cultures involving fungi and embryonic stadium of their host plant tissues may provide the basis to the evaluation of the pathogenicity and severity of hazard caused by these fungi. It may also allow the selection of plant genotypes that are more resistant to certain pathogens, which is particularly important in the cases of fungi known for their ability of epiphytic occurrence. The selection at the embryonic level of tree genotypes resistant to infection would emerge a new direction in the resistance breeding [14].

The aim of this chapter is to present in a condensed way current issues and results of research on interactions between fungi and the host plant callus in dual cultures. This would bring the attention to the usefulness of this method to study pathogenicity and to the opportunities of its application in the forest practice.

## 2. Materials and methods

The *in vitro* cultured tree callus is the plant material necessary to establish a dual culture and fungus is the second organism which the dual culture consists of. The fungal material growing in dual culture with callus needs to be isolated and identified. The callus initiation in the case of gymnosperm trees is based on a biotechnological method – somatic embryogenesis, involving the initiation of callus on disinfected primary explant (e.g. zygotic embryo isolated from a seed). There are also dual cultures, in which two different fungi grow in one culture.

## 2.1. Stages of dual cultures

Dual cultures consist of the following steps:

- Obtaining a considerable amount of non-embryogenic or embryogenic callus of the studied plant species, e.g. by using a somatic embryogenesis method.

The *in vitro* initiation of non-embryogenic (unorganized mass of dividing cells, in nature – cells that cover wounds) or embryogenic (unorganized mass of dividing cells, able to form somatic embryos and in later stages of organogenesis – to regenerate plants in the process of micropropagation) callus in sufficiently large amounts is an essential element to establish a dual culture (Fig. 1). To achieve this objective, an optimal method for obtaining callus (e.g. somatic embryogenesis) should be selected, disinfection of explants should be developed and media together with growth regulators, concentration of sugars and other components necessary for the proper growth of callus should be selected individually for each species [18].

- Selecting the best genotypes (lines) of callus, which should be proliferated until obtaining at least 15 clones of each genotype weighing 300 - 500 mg each [15, 16, 17].
- Isolation and identification of fungi.

Fungi should be properly selected, most preferably with different ecological statuses, corresponding to the studied host plant species. Moreover, they need to be isolated and identified. Traditional methods of fungal identification based on mycelial morphology may be complemented by using molecular techniques based on DNA sequences that allow rapid selection and identification of these organisms.

- Adaptation of fungi selected for dual culture to grow on an “enriched” medium, dedicated to the proliferation of plant callus.

Mycelial fragments of 0.5 x 0.5 cm are transferred from malt extract agar (MEA) onto a medium on which the callus has already been cultured, i.e. the medium with high concentration of macro- and micronutrients and other components (such as casein hydrolyzate, meso-inositol, growth regulators). Fungal identification based on their phenotypic features should be repeated after about 1- 3 weeks.

- Establishment of dual cultures (fungus – plant callus).

Dual cultures are established in sterile Petri dishes with solidified callus-proliferation medium. Fungal inoculum of 0.5 x 0.5 cm should be placed in the center of the sterile Petri dish while the host-plant callus with a diameter of 1.0 - 1.5 cm and weighing 500 mg should be put 5.0 mm from the edge of the dish [19]. Very slow-growing fungi, e.g. *Lophodermium sediciosum* should be placed closer to the callus. Replicates of cultures (their number depends on the frequency of embryogenesis and proliferative capacity of callus genotypes) are stored in the dark in incubators or phytotrons at 25°C +/- 1°C and humidity of 40 – 50%. Moreover, anomalies in callus cells exposed to stress should be microscopically analyzed and immune proteins should be identified. In order to identify the molecular basis for fungal pathogenicity on embryonic level in dual *in vitro* cultures, genetic analyses using molecular markers are

applied. Previously acquired knowledge supported by practice in dual *in vitro* cultures allowed the Author of this paper to draw attention to the aspects of the possible research directions. The analyses may therefore be directed towards: understanding the type of interactions between the tested organisms and assessment of the pathogen behavior under the influence of host plant callus and the callus behavior under the influence of the pathogen, as well as towards the selection of pathogen-resistant plants. Therefore, dual culture experiments may be conducted on one - selected callus genotype or on multiple genotypes, depending on whether we want to select genotypes resistant to the pathogen, or rather to thoroughly investigate the genotype of the pathogen.

- Conducting measurements together with macro- and microscopic observations summarized with statistical analysis.

Species of fungus:	Friedmann's test results:		Average ranks:			
	F	p	G4	G5	G6	Control
<i>H. abietinum</i>	11.846	0.0079*	2.95	1.77	3.14	2.14
<i>H. parviporum</i>	22.773	0.0001*	3.14	2.14	3.41	1.32
<i>H. annosum</i>	17.487	0.0006*	2.27	3.09	3.23	1.41

**Table 1.** Results of Friedman's ANOVA for dual cultures of *Heterobasidion* fungi with the callus of *Abies alba*. \* differences statistically significant at  $\alpha = 0,05$

Species of fungus:	Direction of the mycelium range	Mean	Compared directions of mycelium range	Difference	p*
<i>Heterobasidion abietinum</i>	control	20.28	towards callus - oppositely	- 0.434	
	towards callus	20.76	towards callus - control	0.480	
	oppositely to callus	21.19	oppositely - control	0.914	
<i>Heterobasidion parviporum</i>	control	19.52	towards callus - oppositely	0.070	
	towards callus	24.01	towards callus - control	4.490	+
	oppositely to callus	23.94	oppositely - control	4.420	+
<i>Heterobasidion annosum</i>	control	20.96	towards callus - oppositely	0.029	
	towards callus	22.82	towards callus - control	1.862	+
	oppositely to callus	22.79	oppositely - control	1.939	+

**Table 2.** The statistically significant differences of the fungal growth of *Heterobasidion* species in dual cultures with *Abies alba* callus (towards the callus and oppositely to it) when compared to the control mycelium (t – Student's test for dependent variables). \*statistically significant differences (+) in the growth of the objects compared, with confidence level 95%

Measurements of mycelial growth range are carried out in the directions towards and opposite to the callus. Macro- and microscopic observations of fungi and callus are carried out in

terms of phenotypic changes in tissues with particular reference to anomalies in the callus cells subjected to stress. Measurements and observations are performed every 24 hours (in the case of fast-growing fungi) or every 48 hours (in the case of slow-growing fungi). Based on the results on the growth of tissues, a Friedman's repeated-measures analysis of variance should be performed to verify whether there is a statistically significant difference in the growth of the analyzed fungi towards the examined genotypes (lines) of callus (Tab. 1). To determine inhibition or stimulation of fungal growth it is recommended to perform a t-Student test for dependent variables based on measurements of average length of the mycelial radius (Tab. 2).

- Biochemical analyses:

- identification of PR-type immune proteins (*pathogenesis-related proteins*) in plant callus.

After establishing a dual culture, hyphae grows at different rates towards the living callus tissue. Once the mycelium approaches the distance of about 1-2 mm to the callus or stops its growth at some distance from the callus, the callus tissue should be sampled for the analysis of proteins. The callus may be stored in a freezer at - 81°C, in sterile, 0.5 ml Eppendorf tubes. The next step is the quantitative determination of fresh and dry weight of plants (callus), protein extraction and their quantitative determination using e.g. [1] using bovine albumin as a standard. This method may be modified according to the specific needs. Further biochemical analyses concern separation of proteins depending on their mass (length of polypeptide chain) using vertical electrophoresis in the presence of SDS (SDS-PAGE electrophoresis). The samples need to be properly prepared (buffer optimization, selection of centrifugation time and voltage – usually 10-30 mA/1mm of gel thickness). For proper electrophoretic separation of proteins according to their weight, the samples need to be reduced in appropriate temperature and buffer before applying to gel. Mercaptoethanol or DTT (dithiothreitol) need to be used for this purpose. Both compounds reduce the disulfide bridges (S-S) between proteins, therefore denaturizing the secondary structure of proteins. This procedure is intended to exclude that the heavier but more “packaged” protein migrates faster in the gel than the protein with lower weight but less “packaged”. To obtain good electrophoretic separation, the sample volume must not exceed 10% of the path volume and the amount of proteins applied to the wells of the gel must fit within the range of 10-50 µg. Furthermore, the excessive amount of salt in the sample needs to be reduced, as its ionized form may cause smudging and heterogeneity of bands. Staining of bands on the gel requires selection of the most common staining methods such as using Coomassie Brilliant Blue. The bands are visualized using a gel documentation system with a digital camera. The image of the obtained bands is used to determine the length of proteins with respect to the marker and to establish which of them may qualify as immune proteins produced by the callus tissue.

- introducing elicitors that induce plant defense responses.

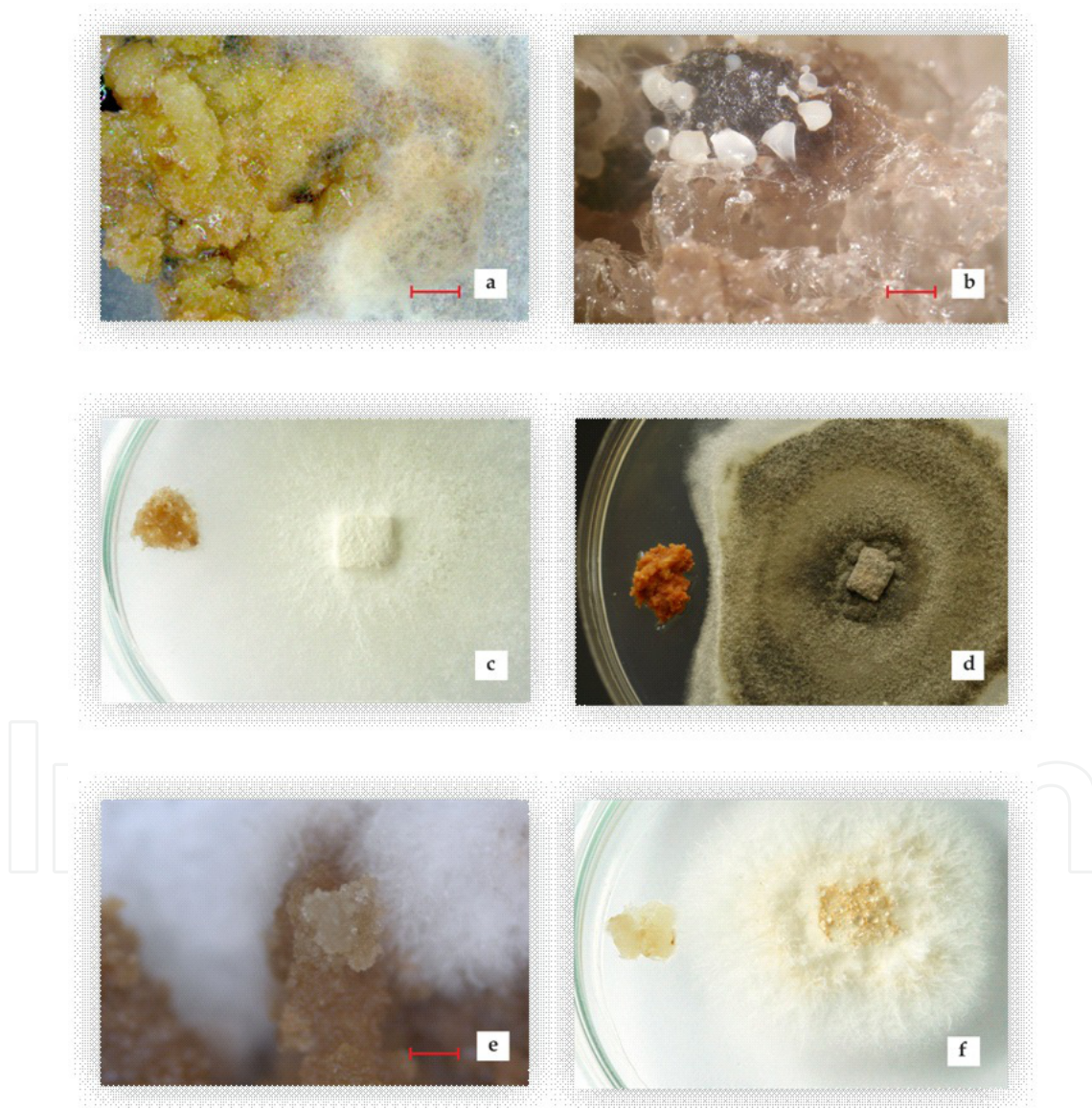
Because of the need to maintain sterile conditions, defense reactions may be induced only with sterile elicitors added to culture media. In such cases specific toxins produced by a pathogen, or post-culture filtrates of the studied fungi are often applied. Mycelia are cul-



tured in liquid aerated media which are subsequently filtered. In this case, we examine the response of tissues or individual cells to the entire spectrum of metabolites excreted by fungi into media. These studies are often based on callus cultures as well as on cell suspensions or protoplasts.

- Inoculation of *in vitro* acquired somatic seedlings with the studied fungus.

When carrying out further steps of the somatic embryogenesis process, after a year of research we obtain somatic plant seedlings on embryogenic callus, which then may be subjected to classical pathogenicity tests.



**Figure 1.** Dual cultures *in vitro* (callus - fungus): a - *Pinus sylvestris* - *Lophodermium seditiosum*, b - *Abies alba* - *Heterobasidion parviporum*, c - *Abies alba* - *Heterobasidion abietinum*, d, e - *Pinus sylvestris* - *Phacidium lacerum*, f - *Abies alba* - *Geosmithia* sp.; Bars a, b, e = 7.0 mm

### 3. The possibilities of using dual cultures

Studies on dual cultures in the world started in the second half of the eighties. At that time the *in vitro* studies were conducted on endophytic fungi and host plant callus. These experiments included both forest trees and herbaceous plants [8, 27]. Analyses of dual cultures in subsequent years involved selected fungal species with pathogenic [3, 29, 13, 2], endophytic [24] and ectomycorrhizal properties [28, 22, 21]. Saprotrophic fungi were most often subjected to studies for comparative purposes (negative control) [3]. In Poland those studies were initiated in 2006 at the Department of Forest Pathology, University of Agriculture in Kraków (Nawrot–Chorabik unpublished). Defense reactions of embryogenic tissues towards various isolates of *Heterobasidion* fungi were studied on the selected genotypes of *in vitro* cultured callus of silver fir. In subsequent years the analyses were supplemented with tests on fungi with different ecological status [19, 20]. The research is continued and cover biochemical, cytological and other aspects.

#### 3.1. Pathogenic organisms

Different organisms were taken into consideration as pathogens in dual cultures: bacteria, fungi and fungi-like organisms (*Chromista*), and were tested with different plants: agriculturally cultivated, fruit trees and parasitic flower plants [2]. Given the chronology of research in dual cultures it may be observed that they gradually become more and more important, which is reflected in the application of more complicated analyses presented by the authors of publications. One of the first research in this field was carried out by [4, 5, 6], who conducted *in vitro* studies of spruce, beech, birch and poplar. They attempted to determine the role of basidiomycete fungi (*Basidiomycota*) in the phenomenon of dying back of some forest tree species. The observed stimulation of fungal growth by the callus was significant for the necessity of studying dual *in vitro* cultures. This phenomenon was the earliest reported in 1981 by [5], who decided to introduce dual cultures into pathogenicity research. These authors showed that wood decay-causing fungi, capable of infecting living tree tissues were stimulated by the callus, while the development of fungi that are able to colonize only dead tissues was inhibited. Based on these results it occurred that the degree of growth stimulation is positively correlated with fungal virulence. [3], taking into account the changes that occur in the forest environment under the influence of different pathogenic and saprophytic fungal species in dual *in vitro* cultures with beech, considered the implications of wider use of such methods for clarifying forest pathology issues. Experiments have shown that the specificity of fungal species towards the particular plant tissue or organ may be better demonstrated using dual systems than with any other methods. It was additionally stated that the dual culture method may be appropriate in the case of forest fungi, because it provides a model of cell division which is similar to the cambium of trees [3]. In the experiment of [13] or [29] the growth of pathogenic fungi was observed in the presence of callus obtained from a few genotypes of Norway spruce (*Picea abies*) and Mediterranean cypress (*Cupressus sempervirens*). [13] demonstrated a clear effect of the spruce callus on the growth of the mycelium. This result was consistent with conclusions drawn by [4]. In the experiments of [13] two genotypes of plant callus differed significantly in their susceptibility to



pathogenic fungi. The growth of *Heterobasidion annosum* cultured with the spruce callus of one genotype was significantly inhibited after 60 hours, while after the same time of culture with another genotype – it was not inhibited. Changes involving the reduction of matter of living callus while increasing the share of dead callus were observed in tissue cultures of sweet chestnut (*Castanea sativa*) affected by the virulent strain of *Cryphonectria parasitica*. In this case, the research in dual cultures fully confirmed the *in vivo* observations [25]. Many studies show that virulent pathogens are stimulated by the callus in dual cultures, while saprotrophic fungi or fungi that re-colonize wood are inhibited by the callus [5, 3, 13, 33]. This phenomenon was previously noted by [5], who decided to introduce dual cultures into the study of pathogenicity, including basidiomycete fungi causing wood decay of living trees. Among fungi occurring on fruit trees, considerable attention was given to the application of dual cultures in the selection of apple trees resistant to apple scab caused by *Venturia inaequalis*. However considering bacteria and fruit trees combination, the majority of studies were focused on *Agrobacterium tumefaciens* - the cause of root galls and *Erwinia amylovora* - the cause of fire blight. On the other hand, among the supergroup *Chromista* the genus *Phytophthora*, which includes the causal agents of phytophthoroses of different trees, shrubs and ornamental plants [23], was mainly taken into consideration. An example of the earliest practical use of tissue cultures in plant protection was the cultivation of virus-free poplar hybrids [23]. The usefulness of dual cultures to evaluate the pathogenicity of fungi was also confirmed with regard to parasitic flowering plants. Hemlock dwarf mistletoe (*Arceuthobium tsugense*) occurring on *Tsuga* was colonized *in vivo* by two fungi: *Cylindrocarpon cylindroides* and *Colletotrichum gloeosporioides*. The dual culture analysis with fungi and the mistletoe tissue showed high pathogenicity of both fungal species, which colonized the plant tissue inter- and intracellularly causing cell walls' degradation [2].

### 3.2. Endophytic fungi

Some of the earliest papers on endophytic fungi in dual cultures were focused on cultures of bigleaf maple (*Acer macrophyllum*) [27] and herbaceous plants: red deadnettle (*Lamium purpureum*) and wood sage (*Teucrium scorodonia*) [24]. The effects of interaction between the endophyte *Cryptodiaporthe hystrix* and the callus of its host – bigleaf maple were studied in dual cultures as mutual interactions. All *C. hystrix* isolates inhibited the growth of callus (the opposite situation was in the case of fungus, whose growth was always strongly stimulated by the callus presence). The Authors suggest that *C. hystrix* and its host (*Acer macrophyllum*) exist in a near-equilibrium state for a certain time, i.e. in natural conditions maple is able to prevent the extensive growth of *C. hystrix* in its tissue. But eventually *C. hystrix* not only did inhibit the growth of callus, but finally overgrew and killed it [27]. This may indicate that *C. hystrix* represents the group of endophytes that may become pathogenic when the plant is under stress conditions. The dual culture analyses confirm the results of the studies conducted *in vivo*, emphasizing that when the plant is under stress conditions (industrial immissions, parasitic insects, adverse weather conditions) and when aging of the plant organs, endophytes may become pathogenic [9, 10, 11]. Studies on dual *in vitro* cultures may contribute to obtaining more detailed results, that provide the basis for determining which species of endophytic fungi are able to cause the tree disease under stress [27]. Among

herbaceous plants, the studies in dual cultures with endophytes were conducted on the host plant and non-host plants (red deadnettle - *Lamium purpureum* and wood sage - *Teucrium scorodonia*) [24]. Endophytic fungi: *Coniothyrium palmarum*, *Geniculosporium* sp. and *Phomopsis* sp. under the influence of the herbaceous host plant callus were characterized by increased growth – as opposed to the behavior of the same fungi in dual cultures with the callus of non-host plants [24].

### 3.3. Ectomycorrhizal fungi

The first research on ectomycorrhizal fungi in dual cultures was initiated in Germany [28] at the Institute of Botany in Tübingen, where Norway spruce (*Picea abies*) callus and mycorrhizal fungi: *Amanita muscaria*, *Lactarius deterrimus*, *Hebeloma crustuliniforme*, *Suillus variegatus* were studied. Pathogenic fungus, that does not stimulate mycorrhization, i.e. *Heterobasidion annosum* was used as control. The results were unambiguous: only two of the studied mycorrhizal fungi (*S. variegatus* and *L. deterrimus*) caused distinct response of spruce cells, which - wrapped with Hartig net - were characterized by better nutrient exchange, especially in the interaction zone of dual cultures. Additionally, irregularities in cell structures of the callus were observed, including the cell cytoplasm, which withdrew in favor of the fungal hyphae. Similar studies were conducted at the Institute of Botany in Finland in cooperation with the Forest Research Institute in Slovakia [22]. The research was conducted on three genotypes of Scots pine (*Pinus sylvestris*) callus and ectomycorrhizal fungi: *Laccaria bicolor*, *L. proxima*, *Pisolithus tinctorius*, *Paxillus involutus* and *Suillus variegatus*. Research in dual cultures was conducted on the effect of these fungi on initiation and proliferation of embryogenic callus [22]. Depending on the fungal species, positive (better callus growth) or negative (browning, necrosis of the callus) embryogenic tissue reaction was observed. Observations of callus cells' behavior under the influence of stress factor (fungi) showed that the hyphae of *Laccaria bicolor* "penetrated" embryogenic cells of Scots pine and by entwining them, it caused positive growth reaction [22]. A few years later [21] retook the research in dual cultures of fungus *Pisolithus tinctorius* and Scots pine. This time it was demonstrated that this fungus improves the *in vitro* germination of somatic embryos of pine. To accomplish this task, somatic embryos of Scots pine, induced *in vitro* on embryogenic callus, were subjected to mycorrhization. The Authors, through multi-faceted research methodology, using a variety of media containing different sugar concentrations and other necessary breeding procedures, obtained the desired effect in dual cultures. Using this method they improved one of the most difficult stages of somatic embryogenesis of Scots pine, i.e. germination of somatic embryos into seedlings.

## 4. Protein as a symptom of fungal virulence

Proteins produced by the host plant cells are important determining factors as to whether plant tissues defend themselves against the pathogen attack, or do not exhibit defensive characteristics. Dual cultures (fungus – plant callus tissues) may be helpful in protein analysis, because *in vitro* conditions are favorable both to research of proteins as well as other

compounds produced by plants under stress conditions (phenols, reactive oxygen forms, carbohydrates). Moreover, *in vitro* studies on embryonic level may be applied to *in vivo* conditions, in which obtaining relevant results seems difficult to achieve, especially since the resistance protein synthesis is observed not only during the pathogen attack, but also during the exposure of plant cells to abiotic stress factors such as drought or frost. Protein analyses were conducted in dual cultures by [27]. It was found that water-soluble metabolites, produced by the bigleaf maple callus could have been responsible for stimulating the mycelial growth. Similar results were presented by [24], who argued that the metabolites, excreted by three endophytes studied by them in dual cultures both with the callus of herbaceous host plant and with the callus of non-host plant, caused necroses and death of callus cells. These metabolites were nonspecific, as they inhibited the growth of not only one of the hosts - *Lamium purpureum*, but also *Lepidium sativum* and *Cantharis fusca*. The current molecular analyses on DNA and protein cooperation are among the most promising research areas. The processes such as transcription, replication and reparation of DNA engage proteins that function as transcription factors (activators, repressors). Because many transcription factors are responsible for regulation of specific groups of genes (e.g. related to cell cycle), the activity of these proteins has a significant impact on the basic cellular processes, such as callus proliferation, differentiation, organogenesis and others, e.g. response to stress factors (including plant pathogens). The fact that the plants show a high degree of specificity in the detection of the pathogen leads to varying degrees of resistance, which involves the entire mechanism of biochemical reactions. Genes trigger the corresponding proteins that are designed to eliminate the pathogen. In recent years studies have been aimed at the so-called pathogenesis-related proteins, which have been described for various plant-pathogen interactions. In such situation we speak of PRs (*pathogenesis-related proteins*), whose functions made them the potential antimicrobial proteins. PRs were determined and identified biochemically for 14 different plant families. The following enzymatic proteins were identified biochemically:  $\alpha$ -1,3 - glucanase (PR-2); chitinase (PR-3, PR-4, PR-8, PR-11); proteinase (PR-6); peroxidase (PR-9) [32]. These proteins, encoded by the corresponding genes, occurred in tissues infected by pathogens [34].

## 5. Phenolic compounds involved in defense reactions

Phenolic compounds are the most numerous group of secondary metabolites with many different properties. Especially phytoalexins (Greek: *phyton* - plant, *alexein* - defend) are of great importance in the immune responses of plants to abiotic and biotic stresses. [30] proposed to refer to the products of higher plants, that are absent or present in trace amounts in healthy tissues, but accumulated in large quantities after the pathogen attack, as phytoalexins. Currently, phytoalexins are defined as low-molecular derivatives of phenols, accumulated in plants under the influence of various stress factors, such as heavy metals, UV light, drought, frost, pathogens, elicitors and fungicides. They are nonspecific chemical compounds, products of the host plant cells and are intended to stop the pathogen growth after the host plant comes into contact with the parasite, and cause defense reactions only in living cells. More-

over, plants resistant and susceptible to the pathogen should differ in the phytoalexin formation rate and defense reaction should be limited only to the affected tissues and their nearest neighborhood. In order to produce alexins, a signal is needed from reactive forms of oxygen. Elicitors, triggering phytoalexins' production, include: arachidonic acid produced by *Phytophthora infestans*,  $\beta$ -1,3- $\beta$ -1,6-hepta-glucoside produced by *Phytophthora megasperma* and protein cryptogein produced by *Phytophthora cryptogea* [26]. The other phenolic compounds exhibiting direct antibiotic effect include chlorogenic acid, which is a strong fungicide, bacteriocide and virucide. Plant tissues which contain higher concentrations of this acid are more resistant to pathogens.

## 6. Callus tissue necrosis as the effect of hypersensitive response to stress factors

The hypersensitive response is the effect of stress applied to e.g. callus tissue growing in dual culture with the pathogenic fungus. It involves killing the plant's own cells infected by the pathogen, therefore the infection is limited and isolated from the healthy tissue, which prevents the replication and spreading the infected cells in the plant organism. The dying back of cells, which results from the hypersensitivity, is accompanied by necrosis of tissues infected by the pathogen (Fig. 1). However, this type of necrotizing involves different program than the necrosis caused by the change in pH, temperature or hypoxia. Both processes - necrosis (localized cell death within a living organism) and apoptosis (programmed cell death) differ in morphological, biochemical and physiological changes. Necrosis consists in a loss of membrane integrity, cell swelling and disintegration of organelles. This leads to inflammation (rapid increase of heat emission by the advantage of catabolic processes over anabolic ones). Necrosis is not associated with the plant development, it does not involve proteases or nucleases, does not require signal transduction or calcium ions and protein phosphorylation. During apoptosis, due to the adverse impact of external conditions, the cell disintegrates into small apoptotic bodies, that retain cell organelles. These bodies are absorbed by other neighboring cells, which is why apoptosis is not accompanied by inflammation [26]. The plant tissue infected by the pathogen triggers a series of active defense-related processes. This is why the mechanism and effect of pathogenesis is complicated. During the defense reaction plant cells may simultaneously launch several signal transduction pathways, which finally results in visible necroses, reflected in the subsequent physiological condition of plants subjected to stress factors.

## 7. Conclusions

Many experiments were carried out using pathogenic, endophytic, ectomycorrhizal or saprotrophic fungi and the embryonic stadium of *in vitro* cultured host plant tissue. Results of these experiments indicated diverse applicability of dual cultures in forest practice. According to the Author of this paper, developing reliable *in vitro* assays may provide a basis for



the evaluation of the pathogenicity and the degree of threat posed by fungi. This may also enable the selection on embryonic level the plant genotype that is more resistant to the pathogen, which is particularly important for fungi known for their ability of epiphytic occurrence. Moreover, the studies of dual *in vitro* cultures may provide more detailed results which would be the basis for determining which species of endophytic fungi are able to cause the disease in trees under stress conditions. Interactions observed in dual cultures may be used to assess the biotrophic properties of various fungal species or lower taxa (strains, varieties, physiological races). These studies may provide the basis for more proper determining of etiology of many diseases and to determine the correlation between the type of protein produced by the callus and the degree of fungal virulence. Conditions, which plant tissues (callus) are subject to during conducting dual cultures induce anatomical changes in callus cells, which may be used in identifying the mechanism of stress factor effect on cyto-physiological changes in plant cells. In the case of positive results of analyses, the dual cultures may give rise to new techniques used in pathogenicity studies in forest pathology.

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