Growth and Development of Bunt and Smut Agents on Wheat Calluses and Availability that Received Co-culture as a Suitable Test-system for the Search of Plant Resistance Inducers

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Abstract: In the recent years the method of tissue culture is actively used in investigations of interaction of plants with fungous agents because as it was shown, the interaction in culture reflects the interaction in nature. Between phytopathogens of wheat *Tilletia caries* (DC.) Tul. and *Ustilago tritici* (Pers.) Jens., that cause bunt and smut, respectively, are unique parasites. These pathogens develop in plant without manifesting of any symptoms during long time. So the investigation of the possibility of these pathogens development on callus cultures is very interesting. For the obtaining the callus tissue not-mature germs of common wheat *Triticum aestivum* cv. Zhnitsa were used. After 10 days of cultivation in second passage calluses were infected by spores of *T. caries* or by mycelium of *U. tritici*. Visual observations have shown on 5th day after inoculation the germination of *T. caries* spores on callus surface. The fungal mycelium was white and downy and formed beige-colored caps in late periods of combined cultivation. The *U. tritici* mycelium was cream-gray-colored, yeast like and grew slower than mycelium of *T. caries* at first time, but then it covered fast all surface of callus. Cytological investigations have shown that hyphae of *T. caries* aerial mycelium were intensively stained and sparingly branched. As growing on callus, aerial mycelium hyphae were interlaced, condensed and in zone adjacent to callus surface mycelium became very dense. On the contrary *U. tritici* hyphae interlaced weakly and disposed mostly regulate in horizontal and vertical directions. During 30 days of combined cultivation not only aerial but also intra-tissue mycelium were observed in wheat calluses. Hyphae of both pathogens colonized 5–7 layers of peripheral cells and were observed both in intercellular space and inside parenchyma-like cells of calluses, but both pathogens did not colonize zones of meristema-like cells and cells of conducting system of rhizoids. Both similarity and difference can be observed in morphological structure of intra-tissue mycelium of *T. caries* and *U. tritici*. The filaments of mycelium were very thin and long, weakly stained, when hyphae of *T. caries* were like sparingly branched filaments with different thickness and part of those (stick-like) were strongly basophilic, and others (beads-like) were weakly stained. When comparing development of bunt and smut agents on common wheat calluses, it can be marked additionally, that during 30 days of combined cultivation the MS medium got brown-dark whereas color of medium remained unchanged in the case of *U. tritici*. It is interesting that during cultivation of these agents spores on MS medium the changes of medium color were similar. Probably it was connected with physiological and biochemical features of investigated fungi. So, the principal opportunity of combined (dual) cultures of wheat calluses with bunt and smut agents was shown. Salicylic acid (SA) influence on morphological pathogen characteristics and on protective callus cell response, connected with hydrogen peroxide production, has been studied. During the cultivation of infected wheat calluses on the medium with MS containing SA, the share of diaminobenzidine colored cells (DAB cells) in fungus growth area increased by 30%, which probably, together with the violation of the pathogen morphology caused deceleration of fungus growth. Thus, the presence of DAB-material in infected cells and the influence of SA show that their protective effect is connected with intensification of H_2O_2 production. The obtained data on oxalate oxidase activity in wheat calluses under the influence of *T. caries* infection and processing with SA proves likeness to defense reactions of plant cell *in vivo* and *in vitro*. The data also disclosed one of the protective mechanisms of a well-known plant resistance inductor – SA.

Keywords: callus; inducers; resistance; *Tilletia caries; Ustilago tritici*; wheat

The bunt and smut agents are unique parasites among wheat phytopathogens. These pathogens develop in plant without manifesting any symptoms during long time. They practically destroy biological yield. In this connection, difficulties in carrying out of experimental work in field conditions as on studying of plants interaction physiology with these phytopathogens, or as on search for environmental safety of new plant protection fungicides are created. The use of traditional chemical plant protection pesticides is interfaced to environmental contamination. It demands search of new environmental safety preparations and ways of struggle with pathogens. Primary search of potential fungicides against bunt and smut agents is especially difficult. The complex performance evaluation of plant protection as a rule should be held in field conditions on greater areas. In laboratory conditions, fungicidity is only tested. The opportunity of studying plant resistance inducing properties in these conditions is limited.

In recent years the method of tissue culture is actively used in investigations of plants interaction with fungous agents. So, the investigation of the possibility of these pathogens development on callus cultures is very interesting. This method opens new opportunities for molecular mechanisms knowledge of plants protection as is known, that in conditions of cellular culture of plant cells interaction with pathogen is similar to the picture of interaction that is in nature.

MATERIAL AND METHODS

Plant material and growth condition. Ripe germs of common wheat *T. aestivum* were used as explants for obtaining callus material. Germs were isolated from seed-vessels and were planted onto the nutrient medium Murashige and Scoog (MS). Before the experiment calluses were cultivated at 26°C in the dark on MS nutrient medium without or with addition of 0.05mM salicylic acid or 1 mg/l chitooligosacharides. Control calli were neither infected nor treated with salicylic acid or chitooligosacharides. 10 days after the second subculture, parts of the calluses were infected by bunt pathogen *Tilletia caries* (DC.) Tul. teliospores or smut pathogen *Ustilago tritici* spores, reproduced on common wheat plants in hothouse. About 80–100 spores constituted infection load per each callus. 20 to 30 days later common cultures of wheat bunt *T. caries* and wheat were grafted onto the fresh nutrient mediums. Plant material was weighed and fixed in liquid nitrogen 3, 6, 9, and 12 days after infection. Each sample comprised four calluses.

The cytological detection of H_2O_2 production. The cytological detection of the hydrogen peroxide generation with oxalate oxidase participation in oxidation the chromogenic substratum 3,3-diaminobenzidine. After 3,3-diaminobenzidine -coloring calluses were fixed in ethanol and acetic acid (3:1) and stained over with methylene blue.

RESULTS AND DISCUSSION

We created long-living co-cultures of wheat callus with bunt *T. caries* and smut *U. tritici* fungi agents.

The spores of bunt agent *T. caries* germinated on the 4th day (Figure 1a) and after 15–20 days fungal hyphae were observed both on a surface, and inside of a callus, being diffuse distributed in intercellular spaces. Cytological investigations have shown that hyphae of *T. caries* aerial mycelium were intensively stained and sparingly branched (Figure 1b). After 30 days of fungi mycelium it was not only in intercellular spaces, but sometimes occupied the cells. As growing on callus aerial mycelium hyphae were interlaced, condensed and in zone adjacent to callus surface mycelium became very dense (Figure 1c). After 30 to 60 days the bunt agent *T. caries* formed the spores which are packed in sori. On wheat calluses they were with precise borders and spores settled down densely in them. It is noticeable on cuts, that new spores have appeared light or dark brown and of nonspherical form. Unlike them, the spores used by us for calluses inoculation, were olive-brown and had correct spherical forms with 14–23 microns in diameter. The similar morphological description of spores received in field conditions was recorded also by other explorers (Azbukina & Karatygin 1995).

It was interesting to find out, whether or not teliospores of the bunt fungi, formed on calluses can infect the wheat. For this purpose new spores have been cautiously removed from co-cultures. These spores have been used for inoculation of wheat seeds and 17% of the infected ears were received in conditions of the climatic camera. The degree of defeat of wheat with new spores quite corresponds to average rate of wheat plants

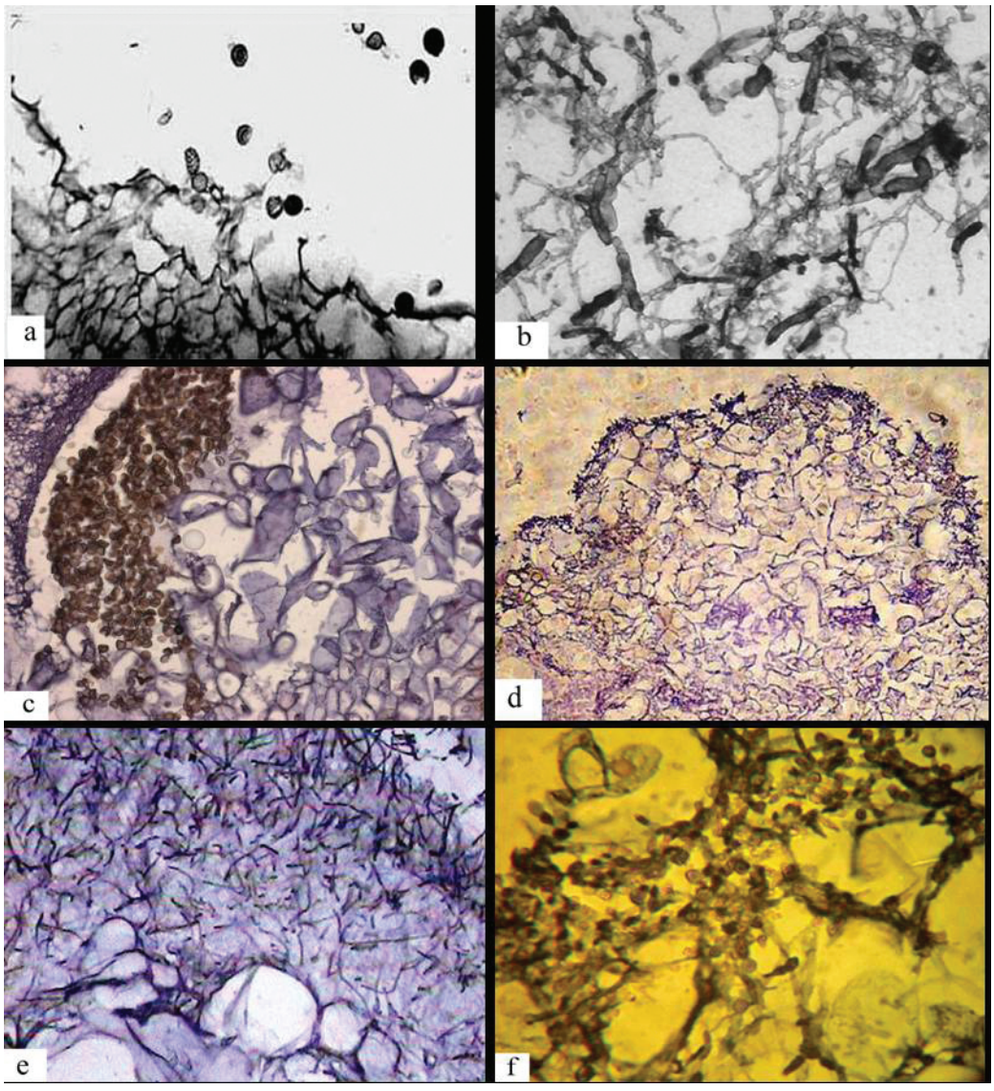

Figure 1. The stages of *T. caries* and *U. tritici* growth and development on wheat calluses:
a – the bunt agent spore germination, × 600; b – stick-like and beads-like fungi hypes of *T. caries*, × 900; c – the bunt fungi sori formation, × 600; d – the smut agent spore germination, × 200; e – intercellular smut mycelium, × 500; f – the new formed smut fungi spores, × 500; a – 4, b – 20, c –45, d – 4, e – 30 and f – 60 days after inoculation

infected with the unspecialized bunt agent form in field conditions. Thus, we have shown an opportunity of long co-cultivating a callus of wheat and *T. caries*. It is revealed, that in co-cultures the pathogen passes a full cycle of development from germination before formation of new spores.

The infecting of wheat calluses with bunt agent led to significant morphological changes in them. So, there was a substantial growth of the calluses sizes at co-cultivation with *T. caries* (Figure 2). The largest cells were observed on periphery of calluses where their close contact with pathogen mycelium occurred. The growth of wheat calluses was accompanied by increase in their crude weight and increase of the size of cells and increase of mitotic index, that we connect with ability of bunt agent to produce phytohormone (Maksimov *et al.* 2002). At calluses co-cultivation with bunt agent, changes in medium on which plant calluses grew were observed. Though fungal mycelium also did not contact growth medium, its coloring in dark brown in process was observed.

Recently, we could receive joint culture of a callus with smut fungi agent *Ustilago tritici*. Vis-

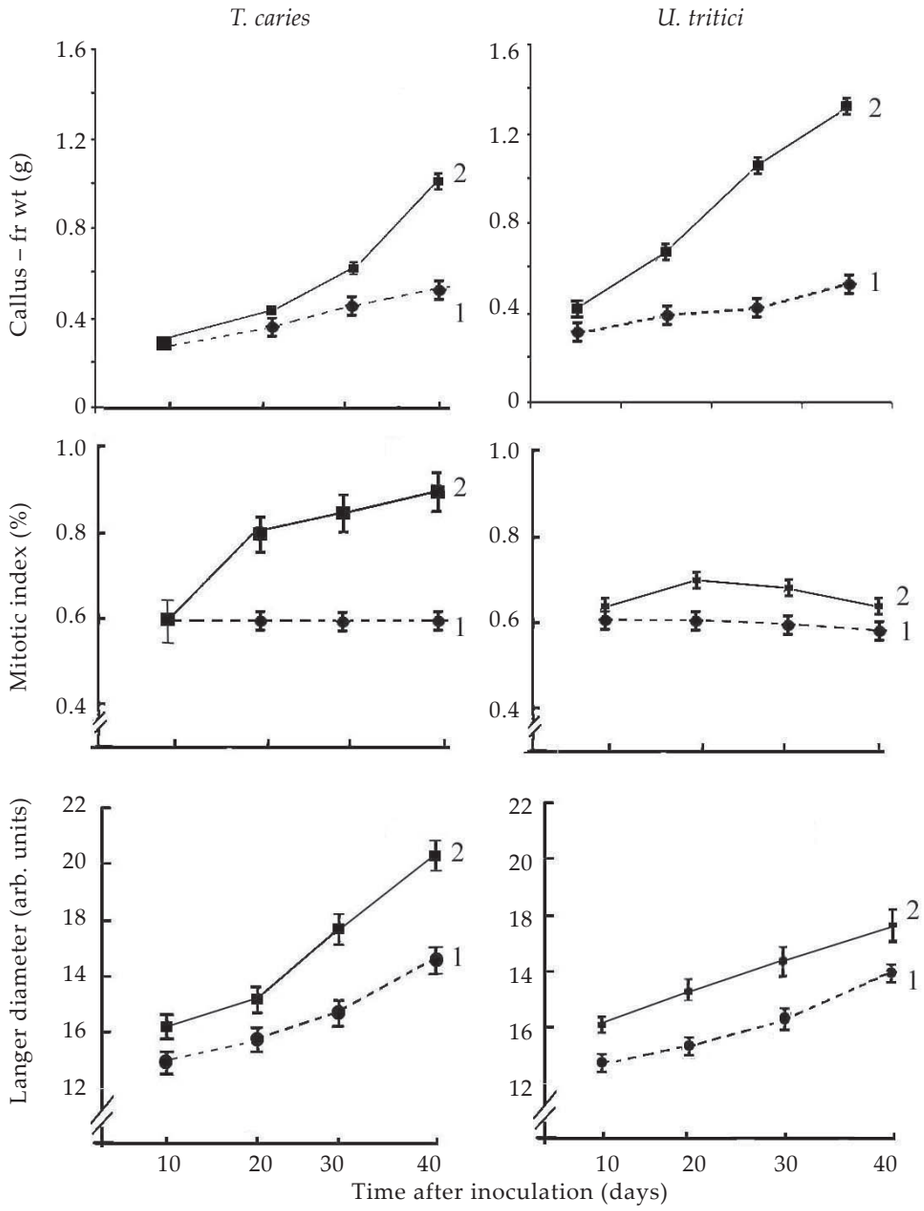

Figure 2. The effect of callus infection with the bunt *T. caries* and smut *U. tritici* agent fungi on fresh weight, mitotic index and cell size: 1 – non infected calluses; 2 – infected calluses

ual observations have shown the germination of *U. tritici* spores on callus surface on the 4th day after inoculation. After 15 days smut mycelium and spores with promycelium were observed and not germinated spores on calluses occurred (Figure 1). The fungal mycelium was visible both on a surface, and inside of a callus. Inside of the callus mycelium has been presented by the hyphae located in intercellular spaces of callus peripheral cells. After 30 days mycelium was observed not only in intercellular spaces of callus periphery cells, but it also occupied cells. Hyphae of smut fungi represented poorly painted long and thin mycelium (Figure 1e).

The *U. tritici* mycelium was cream-gray-colored, yeast like and grew slower. *U. tritici* hyphae interlaced weakly and disposed mostly regulate in horizontal and vertical directions. During 30 days of joint cultivation not only aerial but also intratissue mycelium were observed in wheat calluses.

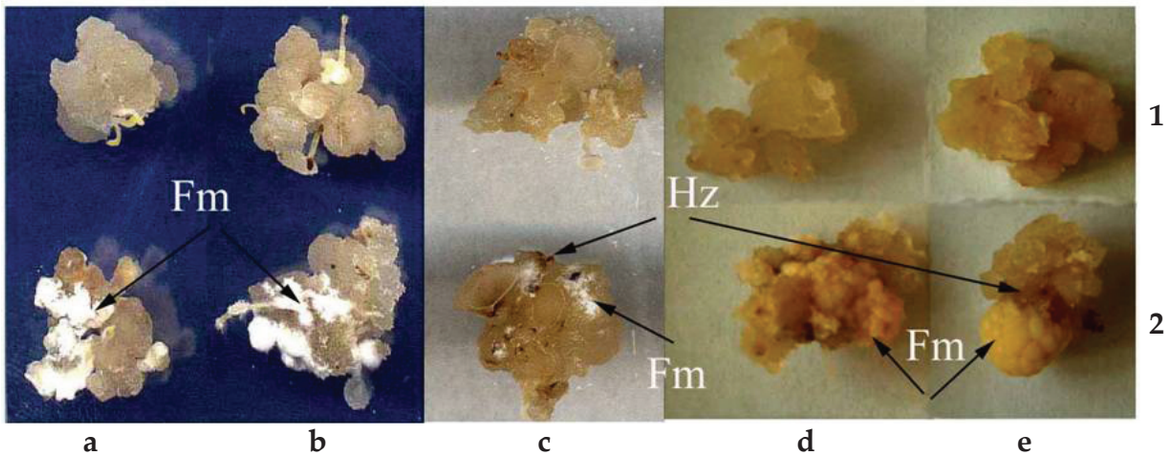

Figure 3. Influence of chitooligosacharides and salicylic acid on morphology and the resistance of wheat calluses in co-culture with bunt *T. caries* (a–c) and smut *U. tritici* (d, e) fungi agents

1 – non infected calluses; 2 – infected calluses: a, d – MS medium; b – MS medium with chitooligosacharides; c, e – MS medium with salicylic acid; 20 day after infection; Fm – fungal mycelium; Hz – hypersensitive zones

It should be noted, that air smut hyphae grew on a surface of a callus almost not intertwining, settling down in order in horizontal and vertical directions (Figure 1e). On air mycelium new spores formed which were found out in the third month. They were brown and their diameter was the same, as of those used for inoculation (Figure 1f).

Similar results were obtained by Kaur *et al*. (1990) in wheat calluses co-culture with carnal bunt agent *Neovossia indica*. To 8–10 day this fungus formed white mycelium in callus which later transformed in yeast-like mucous and in 8 weeks formed spores.

So, hyphae of both pathogens colonized 5 to 7 layers of peripheral cells and were observed both in intercellular space and inside parenchyma-like cells of calluses, but both pathogens did not colonize zones of meristem-like cells and cells of conducting system of rhizoids. Both similarity and difference can be observed in morphological structure of intra-tissue mycelium of *T. caries* and *U. tritici*. The filaments of mycelium were very thin and long, weakly stained, when hyphae of *T. caries* were like sparingly branched filaments with different thickness and part of those (stick-

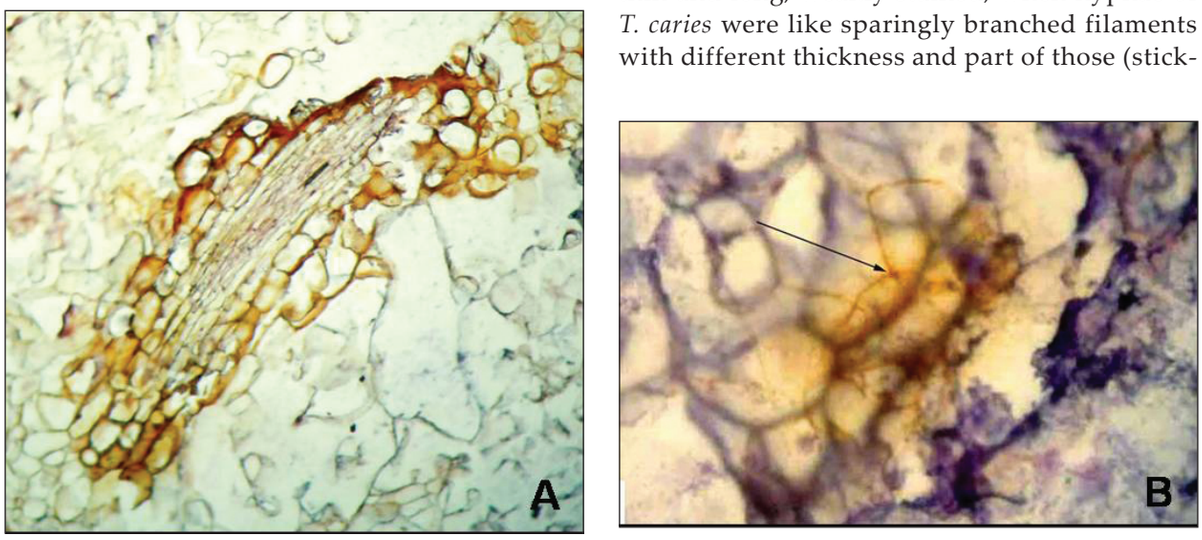

Figure 4. Detection of H_2O_2 in wheat calluses with 3,3-diaminobenzidine around rhizoid of calluses (A) (× 250) and *T. caries* invasion zone (B)

The arrow shows 3,3-diaminobenzidine colored pathogen hyphae in plant callus cells, × 800

like) were strongly basophilic, and others (beads-like) were weakly stained (Figure 1). For a long time (about 3 months after spore inoculation) we observed increase of mycelium and calluses weight. That fact shows that, plant cells and fungi mycelium were viable during term of carrying out of experiments.

Probably, it was connected with physiological and biochemical features of investigated fungi. So, the principal opportunity of obtaining co-cultures of wheat calluses with bunt and smut agents was shown. This culture can be used for studying mechanisms of plant cells resistance to fungal pathogen. As in such system plant cells do not resist to the bunt and smut agents, it can be convenient for an estimation of induction ways of system resistance with various inductors.

Influence of salicylic acid and chitooligosacharides on morphological pathogen bunt *T. caries* and smut *U. tritici* agents characteristics and on protective callus cell response, connected with hydrogen peroxide production, have been studied (Figure 3). The control calluses were large-globular and had a small number of dense sites. Under influence of plant resistance inductors number of dense sites was increased. There was an acceleration of a gain of a biomass of the callus, growing on the medium containing salicylic acid or chitooligosacharides, as well as control calluses in the start of co-culture formation. However in the subsequence gain almost three times decreased and to the end of experiment it did not differ from the gain of control calluses.

There was an appreciable formation of hypersensitivity zones of plant cells and mycelium growth suppression on wheat calluses growing on the medium with a salicylic acid in fungi growth zone (Figure 3c). At calluses growing on the medium with a chitooligosacharides it was not observed. During the cultivation of infected wheat calluses on the medium with MS containing salicylic acid and chitooligosacharides, the share of plant cells to generate hydrogen peroxide in fungus growth area increased by 30–50%, which probably, together with the violation of the pathogen morphology caused deceleration of fungus growth (Figure 4).

Thus, the presence of 3,3-diaminobenzidine colored material in infected cells and the influence of salicylic acid and chitooligosacharides show that their protective effect is connected with intensification of H_2O_2 production. The data also disclosed one of the protective mechanisms of well-known plant resistance inductors.

It is possible to assume, that such character of the calluses response processed by a salicylic acid and chitooligosacharides, on fungi infection is connected with protective action of these means. The fact of a key role of a salicylic acid in induction of local and system resistance of plants and accumulation under its influence of the pathogen induced proteins is widely discussed (Raskin 1992; Alvarez 2000; Metraux 2001).

Besides data were obtained by us for the first time about suppression by a salicylic acid and chitooligosacharides of the cells hypertrophied growth caused by the pathogen. Development of reaction of hypersensitivity of cells testifies to it in reply to infection at the calluses processed by a salicylic acid. This reaction in plants to joint influence and phytopathogens and a salicylic acid observed earlier (Mittler *et al.* 1999; Metraux 2001). Probably, in this system the pathogens play a role of triggers in this reaction (Alvarez 2000), and a salicylic acid a role of secondary messenger (Agrawal *et al.* 2002). These events, as it is known, are the components of the induced system of plants resistance (Metraux 2001). The protective action of chitooligosacharides is also proved and shown in activation of genes coding of pathogen related proteins (Ramonell *et al.* 2002). Thereby in the image, using co-cultures of wheat calluses with bunt agent *T. caries* and smut agent *U. tritici* in work has allowed to visualize its formation and development and to track character changes in intensity of growth processes. The co-culture of wheat calluses with bunt and smut agents was an evident test-system for the search of plant resistance inducers.

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