ABSCISIC ACID AND ETHYLEN INFLUENCE ON ENDO-1,4-B-GLUCANASE ACTIVITY IN TRICHODERMA REESEI I-27

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ABSTRACT
Abscisic acid (ABA) and ethrel (ethylene-producing substance) were applied directly in the inoculum or in the fermentation culture of Trichoderma reesei strain I-27 at 1.0 µM final concentration. ABA provoked 30 % (when applied in fermentation medium) to 10 % (in inoculating suspension) increase of relative endo-1,4-β-glucanase activity (E.C.3.2.1.4) at 72 h of the cultivation while ethrel did not produce any conclusive results. The observed effect in fermentation cultures of ABA treated inoculum was preserved until the end of the cultivation period.

Introduction
The free-living fungi like Trichoderma spp. are very common in soil and root ecosystems. They are opportunistic avirulent plant symbionts (9). Some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cells below this level. They produce or release a variety of compounds. For example xylanase (EIX) from the fungus Trichoderma viride elicits ethylene biosynthesis in leaf tissues of Nicotiana tabacum cv. Xanthi (5). It was established that jasmonic acid/ethylene signaling pathway was involved in the systemic response induced by Trichoderma (26).

Very scarce information about the effect of plant-derived active compounds on Trichoderma physiology is available (12, 14, 27). Influence of phytohormones on different aspects of life cycle and morphology of pathogenic and saprophyte microorganisms, has been an object of several studies (11, 19). The potential applications of host-derived molecules, particularly plant growth regulators, that are able to activate biocontrol and/or enzyme production are very prospective, and could be applied in agriculture as well as in the Trichoderma-mediated industrial production of extracellular enzymes (i.e. they can help to increase the yield and/or reduce the fermentation time) (12).

It has been reported that fungi synthesize abscisic acid (24). Production of ABA in fungi grown under salt stress has been an object of previous work (1, 23).

Both ABA and ethylene are well-known plant stress hormones with growth-inhibiting activities. Studies with different Arabidopsis thaliana mutants have revealed that ABA is important for glucose responses (2, 10). Glucose (Glc) and ethylene signaling pathways antagonize each other in plants (28). How ABA and ethylene exhibit opposite roles in plant Glc responses, and what is the molecular mechanisms underlying the Glc regulation of ethylene signaling and ABA biosynthesis, remain mostly unclear (2, 28).

Cheng et al. (7) provided direct molecular evidence for extensive Glc control of
the genes involved in ABA biosynthesis and signaling. Gene expression analyses also revealed that Glc acted synergistically with endogenous ABA and that Glc regulation was distinct from regulation by osmotic stress. Relationship between sugar signaling and plant growth regulators gives certain reason to examine these phenomena in other organisms as well. *Trichoderma reesei* produces one of the most powerful mixtures of extracellular enzymes for hydrolysis of plant polysaccharides. To our knowledge no data have been published about plant growth regulators’ effects on cellulase production by *Trichoderma spp*. We believed that such study would bring additional information about the control of cellulase expression and its physiological significance.

**Materials and Methods**

Abscisic acid (*cis-trans* mixture) was from Fluka and ethrel™ was a product of Rhone-Poulenc. Tested phytohormones were applied in 1.0 µM final concentration directly in inoculating suspension or in fermentation media.

*Trichoderma reesei* I-27, which was used in the present study, is a variant of the mutant strain M7 obtained by nitroso-guanidine mutagenesis (3, 4).

**Process and Parameters**

Experiment was conducted with 10 days-old culture of *Trichoderma reesei* strain I-27 grown on PDA (potato-dextrose agar) medium at 28°C.

Inoculum was obtained in 500 cm³ flasks which contained 100 ml Mandels mineral salt medium (15) with 2% glucose and 1% maze extract (starting pH after autoclaving 5.8-5.9) for the controls and with added ABA or ethrel (in 1.0 µM final concentration) for the rest of the variants. Inoculating suspension was obtained at 28°C with constant shaking (220 rpm). Fermentation process was held at 28°C with constant shaking (220 rpm). Endo-1,4-β-glucanase (endo-Cx) activity was measured at every 24 h. Cultivation medium was centrifuged at 5000 rpm for 15 min to remove mycelia, and the supernatant was used to test endo-Cx activity.

**Enzyme assay and analytical procedures**

Endo-1,4-β-glucanase activity was determined on sodium carboxy-methyl cellulose (Na-CMC) as substrate according to Wood and Bhat (25). Reaction media containing 0.5 ml 1% solution of Na-CMC in 0.05 M Sodium-acetate buffer, pH 4.8 and 0.5 ml enzyme solution were incubated at 50°C for 30 min.

The cellulase activity was determined by quantifying the reducing sugars liberated during growth according to the Somogyi-Nelson method (21). One unit of cellulase activity (IU) is defined as the amount of enzyme that liberates one µmol of glucose equivalent per minute (50°C, pH 4.8).

Soluble protein was determined by dye binding technique (6) using bovine serum albumin as a protein standard in order to calculate the specific endo-Cx activity.

Results presented are from at least three independent experiments. Although the absolute values of the enzymatic activity measured in respective samples from the separate experiments differed significantly, the trend observed remained constant. In order to obtain interpretable final results endo-Cx activity (in IU) and specific endo-Cx enzymatic activity (in IU/mg total soluble protein) were presented as percentage from the relevant controls.

Data presented are the averages of at least six values (± St. Err.) obtained from three independent experiments.

**Results and Discussion**

Hormone signaling and carbon homeostasis in plants, are tightly coupled and the ar-
Architecture of these interactions is very complex. Plants both produce and utilize carbohydrates and have developed mechanisms to regulate their sugar status and coordinate carbohydrate partitioning (17, 13).

Saloheimo et al. (20) gave basis to seek certain analogy in the regulation of fungal cellulase genes and plant genes by discovering the enzyme swollenin produced by *Trichoderma reesei*. This enzyme probably takes part in liberating a soluble inducer when the fungus is encountering the insoluble cellulosic substrate. Swollenin showed high homology to plant expansins, which are wall-loosening enzymes (8), able to alter the bio-morphological properties of the plant cell walls. According to Saloheimo et al. (20) swollenin gene is homologous to plant expansins and is regulated in a largely similar manner as the *T. reesei* cellulase genes. This finding suggests potential similarities of the regulation of cellulase enzymes and plant cell wall-loosening enzymes or at least such a possibility deserves examination.

Toyofuku et al. (22) suggest a major role for ABA in relation to sugar-signalling pathways, in that it enhances the ability of tissues to respond to subsequent sugar signals. It is generally believed that high levels of exogenous Glc cause ABA accumulation, which results in a delay of germination and an inhibition of seedling development - a typical stress response in plants (18). Considering the existing data on sugar signaling and its relationship with the negative growth regulators in plants, we choose to apply ABA and ethylene during cultivation of *Trichoderma reesei* and to study their effects on endo-1,4-β-glucanase activity. Some of the flasks were inoculated with material grown in the presence of ABA or ethrel with no additional application of the respective compound during the fermentation step.

Influence of exogenous abscisic acid and the ethylene-producing substance ethrel over endo-Cx activity, has been studied at 24 - 120 h of submerged cultivation. In order to evaluate specificity of the effects of both substances, data for relative Cx-activity (represented as % difference from the control) was calculated on Cx-enzymatic activity (in IU) (Fig. 1a, Fig. 2a) and on specific Cx-enzymatic activity (in IU /mg total soluble protein) detected in the cultivation mixture (Fig. 1b, Fig. 2b).

Abscisic acid tended to increase the relative endo-1,4-β-glucanase activity in the middle of the experimental period (72 h) (Fig. 1a, b). The positive effect differed in the two experimental models. It was more permanent in cultures inoculated with 1µM ABA treated suspension (Fig. 1a, b). Having in mind that ABA is a conventional stress phytohormone, which is able to unlock alternative biochemical pathways and physiological processes in order to cover plant organism against unfavorable environmental conditions (16), it was interesting to observe that it advantaged cellulase production (with approximately 50 % increase compared to the control when represented as specific enzymatic activity – Fig. 1b) in fermentation media where the only C-source was Micricel®, whose assimilation by producer cells is normally hard. Stimulating effect of the phytohormone on specific endo-Cx activity was more moderate (about 20 %) when ABA was added in the inoculating suspension, which consisted glucose (Fig. 1a). The observed differences in the ABA effects on specific endo-1,4-β-glucanase activity when applied in the presence or absence of glucose, suggested that this phytohormone is able to alter cellulase production with diverse strength and this is depended on the C-source available in the cultivation medium. ABA exhibited its stimulating effect on endo-1,4-β-glucanase activity shortly after the application of the substance (at 24 h – 48 h) into the cultivation media which suggested that this plant growth regulator specifically influenced the studied enzymatic activity (Fig. 1a, b). Elevated levels of the
Enzymatic activities in the cultures treated with 1 μM ABA were accompanied with relevant decrease of the total soluble protein content. The observed effects on relative endo-Cx activity was not only due to the changes in the total soluble protein content as the positive effect of the phytohormone was preserved even when the results were represented regardless the specificity (Fig. 1a).

Ethylene is known to promote ripening in fruits by triggering chemical reactions that degrade the pectins of the middle lamella, softening fruit, and thus converting the stored starches and oils into sugars that attract seed dispersers. Regarding these
characteristics of ethylene, an idea emerged that it should be checked for potential involvement in the regulation of cellulase production in *Trichoderma reesei*.

Ethrel did not produce any conclusive results. Nevertheless it has been found that when applied in rich medium where the C-source was glucose (in inoculating suspension - Fig. 2b) ethrel stimulated relative endo-Cx activity with 24% to 12% at measuring points 24 h, 48 h and 72 h. Ethylene effect was basically due to the reduced total soluble protein content in *Trichoderma reesei* cultures when grown in the presence of glucose. The data from fermentation cultures (where the sole C-
source was crystalline cellulose Micricel® treated directly with 1µM ethrel were quite inconsistent (Fig. 2a, b). According to them, ethylene caused negligible increase of endo-Cx activity at 72 – 96 h of cultivation (Fig. 2a, b). At the final measuring points (96 h and 120 h) a trend for increase in the total soluble protein content in both cultures subjected to ethylene application, was observed. This reflected in slightly elevated endo-Cx-activity (Fig. 1a) but the positive effect had no specificity as Fig. 2b shows. A possible explanation of the obtained results could be that ethylene may still influence other activities that facilitate cellulase production or release at the end of the experimental period.

Although not entirely conclusive, the results obtained after ethrel application into the cultivation media of Trichoderma reesei, suggested that ethylene does not affect specifically endo-1,4-β-glucanase activity.

The obtained data about ABA and ethylene diverse influence over cellulase production in Trichoderma reesei, supports the idea for further studies of plant growth regulators’ effect over enzyme production by fungi as they may help to increase the yield or at least to design the fermentation process within desired parameters.

REFERENCES