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GIBBERELLIN BIOSYNTHESIS BY *FUSARIUM MONILIFORME* IN THE PRESENCE OF HYDROPHOBIC RESIN AMBERLITE XAD-2

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Summary. The metabolic pathway of gibberellins (GAs) produced by the mutant strain of *Fusarium moniliforme* 3211 is characterized by a high GA_7 concentration. The optimum time for production of GAs was 240th hour after the start of cultivation. In the presence of hydrophobic macroreticular resin Amberlite XAD-2 added at the 144th hour, the concentration of total GAs increased compared to normal cultivation. The main transformation reaction – 1,2-dehydrogenation of GA_4 to GA_7 in the presence of the hydrophobic sorbent was directed towards GA_4 biosynthesis. The alternative diterpenoid pathway directed towards GA_{13} biosynthesis was highly active under these conditions. We hypothesize that the main reason for metabolic processes being directed in this way is the different adsorption of GAs on resin depending on their polarity. The changes of GA concentrations in the presence of hydrophobic resin disturb the equilibrium of enzymatic reactions, mainly 1,2-dehydrogenation, and direct it towards GA_4 biosynthesis.

Key words: gibberellins (GAs), biosynthesis, *Fusarium moniliforme*, hydrophobic resins, reversible enzymatic reactions, chromatography

Introduction

Several species of fungi for example *Gibberella fujikuroi* and *Sphaceloma manihoticola* produce gibberellins (GAs). According to reviews of Bearder (1983) and Graebe

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(1987) the metabolic pathway for GAs biosynthesis starts from mevalonic acid, through some diterpenes to *ent*-kaurene and then to GA_{12} 7-aldehyde in both higher plants and fungi. The following conversion of C_{20} to C_{19} GAs and some transformation reactions may vary in different organisms. 3β-hydroxylation occurs in *G. fujikuroi* at the level of GA_{12}-7-al or GA_{12}. 13α-hydroxylation affects C_{19} GAs (Graebe et al., 1980). The main intermediates are GA_4, GA_7 and GA_1. The principal final product of *F. moniliforme* (imperfect stage of *G. fujikuroi*) is GA_3. There are many branches from this general pathway, for example leading to the biosynthesis of GA_9, GA_{13}, GA_{25}, GA_{57}, GA_{16} and other GAs (Bearder and MacMillan, 1973; Bearder, 1983). An early hydroxylation is catalysed by microsomal monooxygenases while the later ones are performed by soluble oxygenases in higher plants (Albone et al., 1989). The interest to these enzymes is considerable, although there are difficulties connected with their purification (Kwak et al., 1988; Lange and Graebe, 1989).

Reactions catalysed by 1,2-dehydrogenase connected with conversion of GA_4 to GA_7 have not been characterized in detail. From a logical point of view the intensity and direction of these reactions depend on the components of the cultivation medium, growth of the strain, and concentrations of intermediates. For example, hydroxylation processes like steroid transformation are affected by the presence of co-factors (α-ketoglutarate), ascorbate, and Fe^{3+} ions (Hedden and Graebe, 1982). According to our previous work the intensity of the process depends on the kind of C- and N-sources, initial pH and temperature of cultivation (Bojkova et al., 1991).

The aim of this report was to study GAs biosynthesis during the cultivation of *F. moniliforme* 3211 in the presence of the macroreticular resin Amberlite XAD-2.

Materials and Methods

The strains used in the study were *F. moniliforme* IM and the mutant strain *F. moniliforme* 3211 obtained by γ-ray irradiation (Gancheva, 1996). The mutant strain was characterized by enhanced biosynthesis of GA_4 and GA_7.

The strains were grown in a shake culture (220 rpm) at 28 °C, each flask contained sterile medium (50 ml) as described previously (Bojkova et al., 1991). Samples were taken every 24 hour from 72 to 240th h of cultivation. All fermentations were carried out in triplicate and the data for GAs analysis were averaged.

Liquid–solid cultivation

In some experiments, filter sacks contained different quantities of macroreticular resin. Amberlite XAD-2 was added to the medium at the 140th h of cultivation and the process continued to 240 h. Samples were analysed for GA content both in the sorbent and in the cultural filtrate, and the results were presented as a sum in Table 1.

Table 1. GAs concentrations ($mg.l^{-1}$) in the cultural broth of *F. moniliforme* 3211 at the 240th h of cultivation in the presence of different quantities of Amberlite XAD-2

GAs	Resin quantities (g/50 ml medium)					
	0	0.5	1.0	1.5	2.0	2.5
GA_9	14.4	9.1	45.7	108.5	71.0	186.3
GA_4	79.3	57.9	261.4	456.6	458.6	574.3
GA_7	306.4	477.9	274.8	171.4	275.2	256.8
GA_{13}	14.7	30.1	62.6	732.3	531.4	571.1
GA_3-isolactone	47.2	59.1	41.1	57.7	38.6	80.6
GA_1	67.4	34.0	53.4	76.6	81.2	302.2
GA_3	267.4	289.1	343.0	450.8	461.6	369.6

Analysis of GAs

Cultural filtrate (1–5 ml) was applied on a short chromatographic column packed with Amberlite XAD-2. The column was rinsed with water and GAs were eluted with MeOH. The MeOH eluate was treated with diazomethane (CH_2N_2) and purified by column chromatography on silica. GAs - Me-esters were derivatized to trimethylsylil ethers (Rachev et al., 1988). These were quantified using internal standard (phenanthrene – 0.25 $mg.ml^{-1}$). Where sorbent was used during the cultivation, the sacks of Amberlite XAD-2 were transferred to a column, rinsed with water and eluted as above. The eluate was then analysed as above. The results were presented as a sum of GAs concentrations in the resin and the cultural filtrate.

Quantitative chromatography of GAs

GAs were analysed by GC on DB5 WCOT column (25 m × 0.22 mm i.d.), for example in the following conditions: initial temperature 160 °C (5 min), programme rate 4 °C/min, final temperature 240 °C (5 min), carrier gas N_2 (4 ml/min), detector – FID, internal standard phenanthren (0.25 mg/ml) in $CHCl_3$.

Identification of GAs

The GAs obtained in the cultural medium of the two strains were identified by R_f measuring of fluorescent spots on silica gel TLC plates after reaction with H_2SO_4, GC compared with standards and GC–MS. The mass spectra were compared with those from the library of the instrument and available data (Binks et al., 1969).

Chemicals

GA_9, GA_{13}, GA_4, GA_7, GA_1, GA_3 and GA_3-isolactone were kindly supplied by Prof. Takahashi (Japan). Diazomethane was obtained by treatment of diazald. All other chemicals were of analytical grade purity.

Results and Discussion

The mutant strain *F. moniliforme* 3211 was derived from the parent strain *F. moniliforme* IM by γ-ray irradiation. The strategy of selection was published elsewhere (Gancheva, 1996). Analysis of GAs by GC–MS showed that there were no qualitative changes as a result of γ-ray treatment. In all cases GA_1, GA_3, GA_4, GA_7, GA_9, GA_{13}, GA_3-isolactone and GA_{25} were identified together with two unidentified GAs-like compounds. The main feature of the mutant strain was the increased GA_7 concentration as compared to the one of the parent strains. The ratio of GA_4 to GA_7 concentration in the mutant strain was 2.0–2.5 compared to 1.0 in the parent strain. The concentration of GA_3 was slightly lower in *F. moniliforme* 3211 cultivation medium. The quantities of the other intermediates observed in the fermentation broth of *F. moniliforme* 3211 were unchanged.

Changes in GAs concentrations during the cultivation of *F. moniliforme* 3211

We observed two maxima of GA_4 and GA_7 concentrations at 96 h and 272 h during *F. moniliforme* cultivation. GA_3 concentration increased at 144 h and maximal quantities were observed in the interval between 216 and 264 h. The alternative pathways leading to conversion of GA_4 into GA_1 and GA_{12} into GA_9 and GA_{13} were not intensive and the concentrations of these metabolites were in the region from 10 to 250 $\mu g.ml^{-1}$. The results for GA_3, GA_4, GA_7, GA_{13} and GA_1 concentrations during the cultivation of *F. moniliforme* 3211 are shown in Fig. 1.

Sorption of GAs on Amberlite XAD-2 resin

The sorption of GAs on hydrophobic resins such as Amberlite XAD-2 during the *F. moniliforme* cultivation depended on the quantity of the sorbent under conditions of continuous mass exchange. For example 0.5 g resin in 50 ml broth absorbed 63.1%, 59.4%, 44.7% and 14.9% from GA_9, GA_4, GA_7 and GA_{13} respectively. Use of 1.5 g resin resulted in the sorption of 82.3%, 98.3%, 77.8% and 98.9% from the same GAs. The lowest quantities absorbed were GA_1 and GA_3 (49.0% and 67.9%) using 1.5 g resin. The use of 2.0 and 2.5 g resins for 50 ml broth did not follow the same logical regularity (Fig. 2).

The conditions for GAs sorption on hydrophobic resins such as Amberlite XAD-2 during the cultivation were: temperature 28 °C, continuous mass exchange by shaking at 220 rpm and pH 4.2–4.5. GA_4, GA_9 and GA_{13} were absorbed nearly quantitatively to hydrophobic centres of the resin and their concentrations in the fermentation broth were insignificant. GA_7 was absorbed to a lesser extent compared with GA_4 and GA_9. Absorption of GA_1, GA_3 and GA_3-isolactone was lower (approximately 50%). Obviously the degree of GAs sorption depends on their polarity and the quantity of resin used.

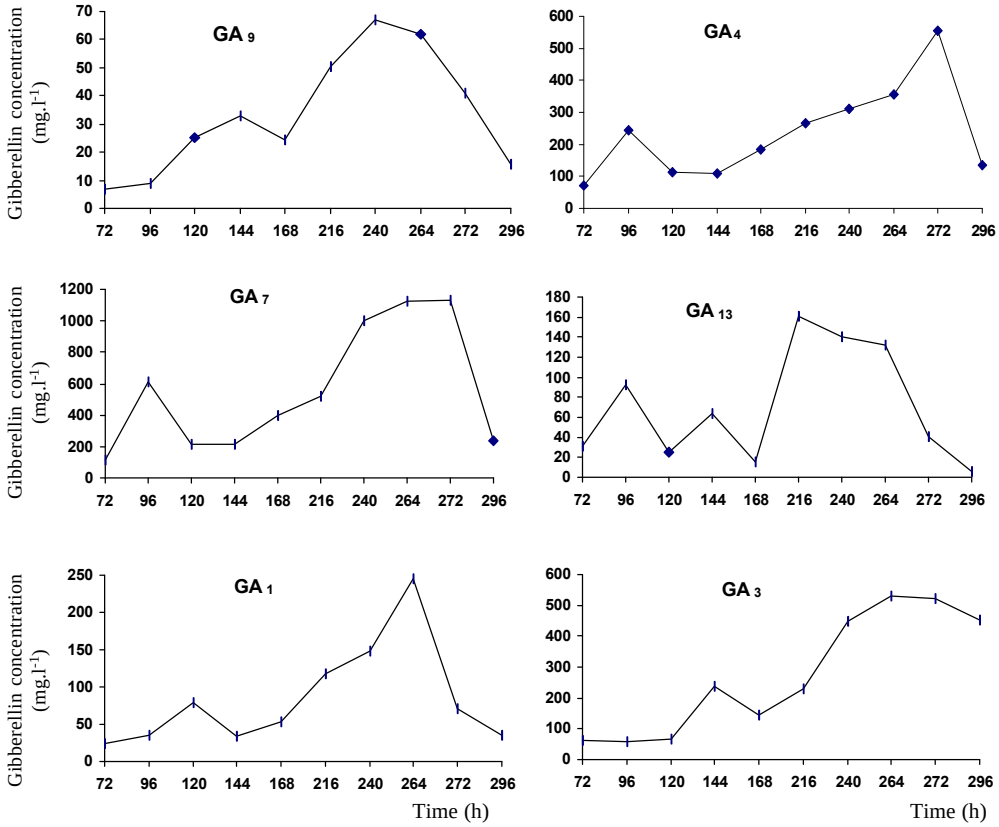

Fig. 1. Biosynthesis of some gibberellins during cultivation of *F. moniliforme* 3211

Influence of Amberlite XAD-2 resin on biosynthesis of GAs by *F. moniliforme* 3211

The use of hydrophobic macroreticular resin Amberlite XAD-2 led to an increase in the total GA concentration. The ratio between GA concentrations in samples to which resin was added at the 140th h of cultivation and samples without resin was 1.46 (0.5 g/50 ml broth), 1.65 (1.0 g/50 ml), 3.13 (1.5 g/50 ml), 3.49 (2.0 g/50 ml) and 3.57 (2.5 g/50 ml). According to this indicator the highest increase in concentration was observed for GA_4 at the 240th h of cultivation. The quantity of GA_7 decreased in the presence of hydrophobic resin and the ratio GA_4/GA_7 was 2.66 when using 1.5 g resin per 50 ml broth compared to 0.26 in the samples without resin.

GA_{13} was formed in relatively low concentrations during the cultivation of *F. moniliforme* 3211. In the presence of hydrophobic resin the concentration of this intermediate product of the minor pathway of GA biosynthesis increased very sharply (49.8 times) at the 240th h of cultivation by use of 1.5 g resin.

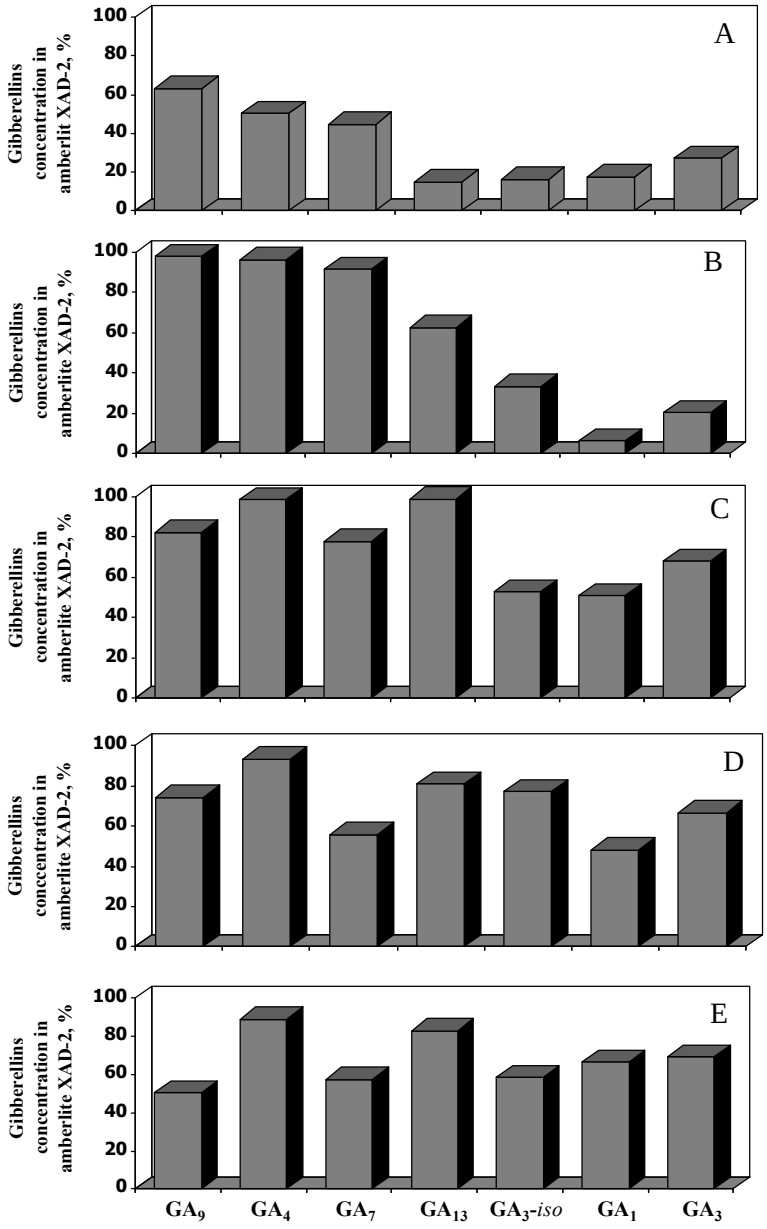

Fig. 2. Sorbtion of gibberellins on Amberlite XAD-2 resin during the cultivation of *F. moniliforme* 3211 (% of total concentration). A – 0.5; B – 1.0; C – 1.5; D – 2.0 and E – 2.5 g resin added to 50 ml medium at the 144th h of the cultivation

GA_1 concentration increased only 1.14 times by use of 1.5 g resin per 50 ml broth, but 4.5 times by use of 2.5 g. It could be mentioned that the sorption of GA_1 in the later case was 66.2%, compared to 55.0% in case of the first one.

GA_9 increased it concentration 12.9 times (2.5 g resin for 50 ml broth) compared with the samples without resin.

According to our results the concentration of GA_3-isolactone was not influenced by the presence of hydrophobic resin in the cultivation medium.

GA_3 increased in concentration in the presence of hydrophobic resin but not so dramatically – approximately 1.8 times more (2.0 g resin for 50 ml broth) compared to samples without resin.

Strain *F. moniliforme* 3211 was selected because it increased GA_7 production. γ-ray irradiation generally did not affect the genome and did not influence the qualitative composition of GAs. As known the main metabolic product of *F. moniliforme* is GA_3. *S. manihoticola* produces only GA_4 due to the absence of 1,2-dehydrogenase and 13α-hydroxylase enzymes responsible for GA_7, GA_1 and GA_3 biosynthesis (Graebe et al., 1980). In contrast, these enzymes are present in *F. moniliforme* 3211 and in *F. moniliforme* IM. The reason for the quantitative changes of GA_4, GA_7 and GA_3 concentrations could be genome damage leading to different morphology, growth requirements or decrease in 13α-hydroxylation activity. According to these data 1,2-dehydrogenase activity in the mutant strain is higher and 13α-hydroxylation activity is lower compared to the parent strain, resulting in larger quantities of GA_7. This is the reason for incomplete GA_7 conversion to GA_3.

The alternative minor pathways such as transformations to GA_1, GA_9 and GA_{13} were not so intensive. Other intermediates observed as GA_{47}, GA_{56}, GA_{16} and GA_{57} were not identified in *F. moniliforme* 3211 and IM by GC–MS analysis (Hedden and Graebe, 1982; Graebe, 1987).

According to our results the sorption of GAs depends on the polarity of the compounds and location of some specific functional groups. Low polarity compounds such as GA_4, GA_7 and GA_9 were nearly quantitatively absorbed to hydrophobic centres of the resin and their concentrations in the fermentation broth were insignificant. The cultural medium pH in the region of 4.0−4.5 decreased the dissociation of acid groups and correspondingly the polarity of carboxylic acids present in the medium. Because of this the sorption of tricarboxylic acids such as GA_{13} was comparable to that of GAs with low polarity such as GA_9 and GA_4. GAs with two hydroxyl groups in the 3β and 13α-positions (GA_1, GA_3 and GA_3-isolactone) were absorbed less by the resin. Double bond in the 1,2-position increased the polarity of GAs to a lower extent than the hydroxyl groups. These could be seen in Fig. 2. Increasing the resin quantities above 1.5 g per 50 ml medium did not improve GA sorption significantly with the exception of GA_1.

Resin was added at the 140th h of the cultivation. Trials in which resin was added earlier led to disturbances in growth and to production of cells that had an abnormal morphology.

The use of two phase liquid–solid fermentation by adding macroreticular resin Amberlite XAD-2 at the 144th h of culture led to an increase of total GAs concentration. This effect was obvious when the resin quantities added were equal or above

1.0 g/50 ml medium. According to our results, intensification of biosynthesis of different GAs depends on the sorption of a specific gibberellin to the resin. The reduction of GA concentrations with higher quantities of resin could be explained by lower growth of fungi, partial inhibition of some enzymatic processes, because of the sorption of some co-factors and activators and lower absorption of GA_4 in this case (Fig. 2). The biosynthesis of GA_7 and GA_3 results from enzymatic reactions of 13-α-hydroxylation and 1,2-dehydrogenation. Obviously the rate of 1,2-dehydrogenation depends on the concentration of the first metabolite in the medium – GA_4. In the presence of hydrophobic resin this concentration decreases significantly probably due to the lower polarity of GA_4 compared with GA_7 and GA_3. Our hypothesis is that, when the concentration of GA_4 in the medium is minimal, the ratio of GA_4 to GA_7 is disturbed and the opposite reaction directed to GA_4 is observed. The main pathway in this case is conversion of available GA_7 to GA_4 catalysed by 1,2-dehydrogenase or its conversion to GA_3 (13β-hydroxylase). The explanation that quantitative sorption of GA_4 stops the process preventing further metabolism probably is not correct because it is not clear why the concentrations of GA_4 and GA_3 were increased in this case (Table 1). Our results show that probably the direction of general biosynthetic pathway from GA_4 via GA_7 to GA_3 is opposite and the consecutive enzymatic reactions mentioned above are reversible in the presence of the hydrophobic resin. The reason is the lower polarity of GA_4 compared to that of GA_7 and GA_3. When the concentration of GA_4 in the liquid phase is minimal the equilibrium of the enzymatic processes is disturbed and the opposite reactions could be observed. The main pathway in this case is conversion of GA_7 to GA_4 catalysed by 1,2-dehydrogenase. The 13α-hydroxylation taking part in the conversion of GA_7 to GA_3 and independently of GA_4 to GA_1 is not affected so much. The reason for a smaller increase in GA_3 concentration is the lower level of GA_7 in the presence of the hydrophobic resin.

GA_{13} is formed in low concentrations by *F. moniliforme* 3211 and IM. In the presence of 1.5 g resin per 50 ml broth the concentration of this minor pathway of GA biosynthesis is increased very sharply. We supose that the high sorption capacity of Amberlite XAD-2 to GA_{13} (Fig. 2) probably led to intensification of biosynthetic pathway – oxidation of GA_{14} via GA_{37}-hydroxyacid and GA_{36} to GA_{13}. The same may be the case with GA_{25} but we did not possess a suitable standard for its quantitative measurement.

Another intermediate, GA_9 is of low polarity and increases in concentration compared with samples without resin. It means that the reactions from GA_{12}-7-al via GA_{12}, GA_{24} to GA_9 are reversible too and the direction depends on the sorption of the final metabolite.

According to our results the concentration of GA_3-isolactone is not influenced by the presence of hydrophobic resin. Possibly GA_3-isolactone is the product of non-enzymatic isomerization of GA_3 in the cultivation medium. This indicates that kind of resin influences only the reversible enzymatic reactions running in the cultural broth.

GA_1 increased in concentration only when the quantity of resin in the cultural medium was above 2.0 g per 50 ml. The maximal sorption of this metabolite was in the presence of 2.5 g resin per 50 ml medium (Fig. 2).

Relatively pure MeOH eluates from Amberlite XAD-2 resin could be obtained as a result of two phase liquid–solid cultivation of *F. moniliforme* 3211. These eluates were enriched mainly with GA_4 and GA_{13}.

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