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SHORT CONTRIBUTION

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Improved GA_1 production by *Fusarium fujikuroi*

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Abstract Recent studies into gibberellin A_1 (GA_1) showed it to be physiologically more active than GA_3 in plants of great agricultural interest, such as tomatoes, rice, peas, and sweet cherries. We describe here a simple procedure for obtaining large quantities of GA_1 (1,500 mg/l) by incubating the FKMC1995 strain of *Fusarium fujikuroi* in a standard complex medium (SCM). We also compare the GA production of this strain with that of two other wild-type strains of *F. fujikuroi* (IMI58289, m567) in SCM and low-nitrogen medium and discuss the possible biogenetic mechanisms involved in the over-accumulation of GA_1 by FKMC1995.

Introduction

Gibberellins (GAs) are phytohormones which occur at trace levels in higher plants and play a part in many important functions in their development (Arteca 1995; MacMillan 1997). There are more than 100 different GAs described so far, amongst which GA_3 is generally considered to be one of the most active (MacMillan 1997). When applied exogenously, GAs produce remarkable responses in both seeds and plants and, in this context, GA_3 and a mixture of GA_4 and GA_7 find diverse applications in agriculture and brewing (Takahashi et al. 1991; Mander 1992). Recent reports, however, described how the biological activity of GA_1 is even higher than that of GA_3 in tomatoes (Grünzweig et al. 1997), rice (Kobayashi et al. 2000), peas (Yaxley et al. 2001), and sweet cherries (Huanpu et al. 2001). The fungus *Fusarium fujikuroi*, corresponding to the mating group C of the *Gibberella fujikuroi* species complex (O′Donnell et al. 1998), is the only organism capable of excreting GAs in industrially viable quantities (Takahashi et al. 1991). To date, 14 different strains of *F. fujikuroi* have been reported as giving relatively high yields of GA_3, the final product of the fungal GA pathway (Voigt et al. 1995); and several procedures for obtaining GA_3 (Birch et al. 1960; Redemann 1959) and a mixture of GA_4 and GA_7 (Jinhu and Fanggui 1999; Lee and Gallazzo 2001) via fermentations of this fungus have been patented. In contrast, little information has been published concerning the production of GA_1 in high enough quantities to be viable for industrial exploitation.

To find out more about the biosynthesis of GAs by *F. fujikuroi*, we previously studied the metabolites excreted by the parent strain IMI58289 and its *gib* mutants SG121, SG136, SG138, and SG139 (Barrero et al. 1992, 1993, 1999, 2001; Fernández-Martín et al. 1995). The aim of our present work was to improve GA production by comparing three different wild-type strains (IMI58289, FKMC1995, m567), employing simple incubation methods. We paid special attention to GA_1, a GA which is so far not commercially available but which holds great potential promise in agriculture.

Materials and methods

Organisms

F. fujikuroi m567 (Culture Collection Weimar, Germany), IMI58289 (Commonwealth Mycological Institute, London, England) and FKMC1995 (Leslie 1991; Kansas State University Collection, Manhattan, Kan., USA).

Media and culture conditions

F. fujikuroi was kept on Sabouraud agar slants at 4 °C. Fragments of mycelium taken from the stored agar slants were transferred to Petri dishes containing sporulation agar and incubated for 6 days at 28 °C in the dark. Sporulation agar for IMI58289 contained (in 1 l

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distilled water): 1 g glucose, 1 g L(+)-asparagine monohydrate, 1 g KPO_4H_2, 0.5 g $MgSO_4 \cdot 7H_2O$, and 16 g agar. Sporulation agar for FKMC1995 and m567 contained (in 1 l distilled water): 30 g glucose, 3 g L(+)-asparagine monohydrate, 1 g KPO_4H_2, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 2 ml trace-element solution (Avalos et al. 1985), and 16 g agar. Suspensions (1 ml) of mycelium fragments and spores, obtained by washing the surface of each agar plate with 20 ml of sterile distilled water, were used to inoculate 500-ml Erlenmeyer flasks containing 50 ml of liquid medium. Low-nitrogen (LN) medium was the ICI medium described by Geissman et al. (1966), in which 4.8 g of NH_4NO_3 in 1 l was replaced by 0.96 g of asparagine. Standard complex medium (SCM) was described by Brückner and Blechschmidt (1991). Incubations were made at 28 °C with orbital shaking at 200 rpm.

Analytical methods

At the end of each incubation, the mycelium was separated from the medium by vacuum filtration. The culture broth was acidified to pH 2 with 2 N HCl and then extracted with EtOAc. The acidic extract was treated with ethereal CH_2N_2 and submitted to column chromatography on silica gel to separate the fatty-acid methyl esters (hexane:*t*-BuOMe, 7:3) from GA methyl esters and other metabolites (*t*-BuOMe). An aliquot from the GA mixture was treated with Sigma-SIL-A to obtain trimethylsilyl derivatives and analyzed by GC-MS, using a HP-5890-A apparatus equipped with a HP-1 column (cross-linked methyl siloxane, 25 m long, 0.2 mm diameter, 0.33 μm film thickness) connected to a HP 5972 mass EI detector at 70 eV. The carrier gas was helium (inlet pressure 105 kPa) and the injection volume for the sample was 1 μl. The temperature of the injector was 260 °C and the temperature program was: from 120 °C to 220 °C, rising by 5 °C/min, then rising by 3 °C/min up to 280 °C, where it was held for 10 min. Compounds were identified by computer search using the NBS75K library of mass spectral data and comparing the mass spectra with published data (Gaskin and MacMillan 1991) and our own standards (Barrero et al. 1992, 1993, 1999, 2001). Quantitative analysis was made on the basis of the area under the peaks of the GC-MS chromatograms. Column chromatography on 20% $AgNO_3$/silica gel (hexane:*t*-BuOMe, 1:9) of the GA mixture obtained from the FKMC1995 strain allowed us to isolate GA_1 and GA_3 (as methyl esters); and their chemical structures were confirmed by ^{13}C NMR spectroscopy (Mander 1992).

Results

It is well known that high concentrations of nitrogen in the culture medium repress GA biosynthesis in *F. fujikuroi* (Brückner and Blechschmidt 1991; Candau et al. 1992). With this idea in mind, we initially resorted to the usual two-stage incubation procedure (Barrero et al. 1999, 2001). This procedure, however, might prove to be quite troublesome, especially when studying various strains incubated in different media or working on a medium-to-large scale. Therefore, we decided to assay a simpler incubation procedure in only one step, by inoculating the fungus directly into minimal liquid medium (Geissman et al. 1966) containing only a low proportion of asparagine as nitrogen source (LN medium) and maintaining the mycelium in this broth for 2 weeks. To gather information about GA production under these novel conditions, we made time-course experiments, incubating the IMI58289 and FKMC1995 strains. The results showed that both strains grew satisfactorily, reaching maximum growth on day 10 of incubation, but IMI58289 produced a higher biomass (8 g mycelium weight/l versus 5 g/l for FKMC1995). Moreover, among the metabolites excreted, we identified not only the GAs GA_1, GA_3, GA_4, GA_7, GA_9, GA_{13}, and GA_{25}, but also the related terpenoids 1–9 (Fig. 1). The IMI58285 strain, when incubated in LN medium (columns labeled B in Table 1), began to excrete GAs on day 4 of growth (on previous days only sesquiterpene 9 was detected in the medium), whereas FKMC1995 (columns labeled A in Table 1) started 24 h earlier. Additionally, the total GAs

Table 1 Quantities (mg/l) of gibberellins (GA_x) and related metabolites (*1–9*) accumulated by *Fusarium fujikuroi* strains FKMC1995, IMI58289, and m567 in different media. *A* FKMC1995 in low-nitrogen (LN) medium, *B* IMI58289 in LN medium, *C* FKMC1995 in standard complex medium (SCM), *D* IMI58289 in SCM, *E* m567 in SCM. The data for GA_3 include iso-GA_3. The data for GA_7 include iso-GA_7. For terpenoid 6, the data include the hydrated form of 6

Compounds identified	Days and media																	
	3	4		5		6		7		8		9	10	11	12			13
	A	A	B	A	B	A	B	A	B	A	B	C	C	C	C	D	E	C
GA_1	3	10		5	2	9	3	8	4	8	6	838	1,401	978	1,594	785	623	1,389
GA_3	33	107	32	54	53	226	74	199	91	173	126	597	708	682	1,005	902	902	1,009
GA_4	3	9	5	7	5	8	6	9	7	8	8	118	135	118	131	Trace	Trace	149
GA_7	9	25	8	13	12	40	20	27	26	24	25							
GA_9		2	2		3		5	3	6	3	6	Trace	24	Trace	Trace			
GA_{13}		17	5	6	10	38	15	38	19	36	28	231	358	261	231	81	55	219
GA_{25}					1		3	3	5	2	6							
GA_{36}												77	107	85	96	Trace	Trace	96
1		18		11	9	46	24	30	35	47	25							
2							1		2					Trace				
3					2	5	3	4	3									
4	6	30	10	10	14	35	19	41	26	30	26		246					204
5	5	12	2	7	4	25	7	22	10	18	13	80		66	68	29	24	
6	7	48	13	21	31	74	34	60	52	79	61	500	681	531	639	104	62	665
7		4		3	2	9	4	13	1	13	8	58	72	118	193	55	48	168
8						5		3	3				28	25	47	Trace	Trace	63
9	6	4	46	6	37		52	3	46		32							

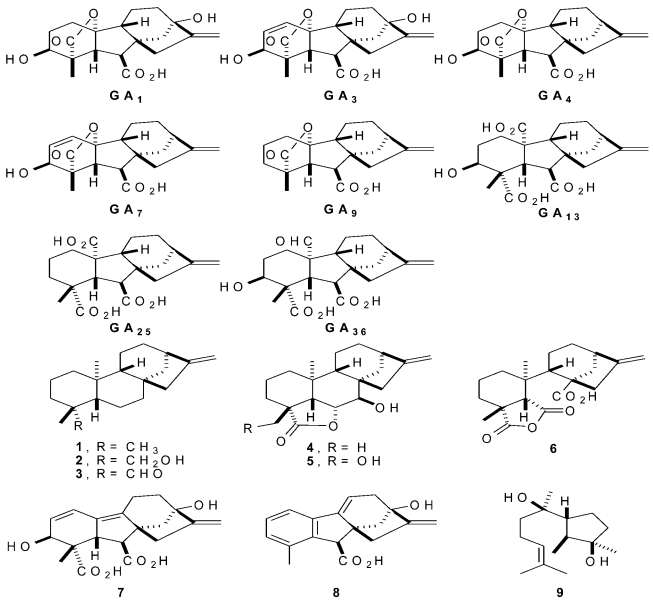

Fig. 1 The gibberellins (GA_x) and related terpenoids (*1–9*) identified among the metabolites accumulated by *Fusarium fujikuroi*

accumulated in the culture broth of FKMC1995 after 1 week of growth (around 250 mg/l) was somewhat higher than that of IMI58289 (roughly 200 mg/l). A third strain, m567, cultured under the same conditions, produced only 130 mg GAs/l.

Since m567 was regarded as one of the wild strains with the greatest capacity for GA_3 production (Voigt et al. 1995), the higher GA production shown by FKMC1995 in LN medium encouraged us to make further experiments with this strain. In 1991, Brückner and Blechschmidt (1991) reported that the m567 strain, when incubated in SCM containing sunflower oil, produced around 1,000 mg GA_3/l, together with lower proportions of GA_4 and GA_7. Nevertheless, in preliminary experiments employing this medium, we detected a noticeable proportion of GA_1, which was not mentioned by those authors. Therefore, we made a time-course experiment with the FKMC1995 strain in SCM (columns labeled C in Table 1). After 12 days of fungal growth, the total GAs accumulated in SCM reached 3,000 mg/l, ten times more than that achieved in LN medium. We were pleased to find that the main product under these growth conditions was GA_1, which constituted half of the entire production of GAs. The high quantities of GA_1 and GA_3 obtained allowed us to isolate them in the form of methyl esters by column chromatography. Interestingly, a pure sample of GA_1 methyl ester, under the conditions of the spectrofluorimetric method used by Brückner and Blechschmidt (1991), gave response intensities eight times lower than those of the GA_3 methyl ester. This phenomenon might go some way to explain why those researchers did not detect GA_1 when they incubated the m567 strain in SCM. In fact, when we incubated the IMI58289 and m567 strains in this medium, we found a considerable accumulation of GA_1 in both cases (Table 1, columns D and E, respectively), but less than with FKMC1995.

To our knowledge, GA_1 production of up to 1,500 mg/l has not been reported previously for any other organism and therefore we believe that the FKMC1995 strain of *F. fujikuroi* is the first fungus described capable of producing sufficient GA_1 for industrial purposes.

Discussion

The capacity of strain FKMC1995 to produce high quantities of GAs adds to its other advantageous biological characteristics to make it a remarkably useful strain. As opposed to strains IMI58289 and m567, the mycelia of FKMC1995 anastomose spontaneously, making the generation of heterokaryons a simple task (J. Avalos, unpublished data). Furthermore, while poor microconidia production and low fertility are frequently associated with GA-producing strains, such as IMI58289 and m567, strain FKMC1995 conidiates abundantly and is sexually fertile against appropriate strains of the opposite sex, such as FKMC1993 (Leslie 1991). In fact, these strains have been used as mating group C tester strains in the *G. fujikuroi* species complex. All these features make FKMC1995 a highly suitable strain for research purposes.

The unusual quantity of GA_1 accumulated in SCM holds hope for potential biotechnological applications and deserves some comment. It might be explained by the presence in this medium of one or more substances capable of acting upon the metabolism of fungal GAs at two different levels: (a) the mechanisms responsible for biosynthesis regulation and (b) the enzyme 1,2-desaturase, which is involved in the penultimate step of GA_3 biogenesis (MacMillan 1997). A derepression of the regulatory mechanisms would promote the overproduction of all GAs, while reduced 1,2-desaturase activity would lead to an accumulation of GA_1, to the detriment of GA_3.

The recent cloning of the genes responsible for GA biosynthesis in *F. fujikuroi* (Tudzynski and Hölter 1998; Hedden et al. 2002) opens new perspectives for research into its regulatory mechanisms. Up to seven genes are found to be tightly clustered in the *F. fujikuroi* genome. Six of them, including the gene coding for the desaturase involved in the conversion of GA_4 into GA_3, via GA_7, share a common regulation by nitrogen, mediated by the transcription factor, AREA (Mihlan et al. 2003). Our results suggest that the reduced desaturase activity observed in SCM should not be attributed to an impaired activation of the gene by AREA, but to a specific inhibition of enzyme activity by the components of this medium. Nevertheless, the GA_1 over-accumulation by FKMC1995 could be imputed not only to the composition of the medium but also to the genetic background of this strain, because this effect is less pronounced in strains IMI58289 and m567.

In conclusion, we describe here a simple procedure for obtaining high quantities of GA_1 (1,500 mg/l) by

incubating the FKMC1995 strain of *F. fujikuroi* in a liquid medium containing sunflower oil (SCM). The biogenetic mechanisms responsible for the accumulation of GA_1 are as yet not fully understood, but may be related to substances contained in the medium and to particular characteristics of the regulation of GA biosynthesis in this strain. We are currently engaged in experiments to identify the components of the medium responsible for the enhanced accumulation of GA_1.

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