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GA_3, ABA and Cytokinin Production by *Lentinus tigrinus* and *Laetiporus sulphureus* Fungi Cultured in the Medium of Olive Oil Mill Waste*

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Abstract: The levels of endogenous form of free, bound and total-gibberellic acid (GA_3), abscisic acid (ABA) and cytokinin (zeatin) in culture medium were determined. The changes in dry weight of the mycelium, dependent on the culture periods, was examined through the use of white-rot fungus *Lentinus tigrinus* and brown-rot fungus *Laetiporus sulphureus* which were cultured in the medium of olive oil mill waste in a static culture. Spectrophotometric techniques were used to determine the amounts of endogenous GA_3, ABA and zeatin.

As a result, fungi used in this study showed that they synthesized GA_3, ABA and zeatin as a primary or secondary metabolite and these plant hormones were found to be in free and bounded forms.

Key Words: *Lentinus tigrinus, Laetiporus sulphureus,* Fungi, Olive mill waste water, Gibberellic acid (GA_3), Abscisic acid (ABA), Zeatin, Plant hormones.

Zeytinyağı Fabrikası Atığında Üretilen *Lentinus tigrinus* ve *Laetiporus sulphureus* Funguslarından GA_3, ABA ve Sitokinin Üretimi

Özet: Statik inkübasyon ortamında ve zeytinyağı fabrikası atığı besiyerinde üretilen beyaz-çürükçül fungus *Lentinus tigrinus* ve kahverengi-çürükçül fungus *Laetiporus sulphureus*'un kültür ortamında kültür periyoduna bağlı olarak serbest-, bağlı- ve total formlarda içsel Gibberellik asit (GA_3), Absisik ait (ABA) ve Sitokinin (zeatin) miktarları ve fungusların kuru misel ağırlıkları saptanmıştır. İçsel GA_3, ABA ve zeatin miktarlarının saptanmasında spektrofotometrik teknik kullanılmıştır.

Sonuç olarak, bu çalışmada kullanılan fungusların hem serbest hemde bağlı formlarda GA_3, ABA ve zeatini birer primer ya da sekonder metabolit olarak sentezledikleri gösterilmiştir.

Anahtar Sözcükler: *Lentinus tigrinus, Laetiporus sulphureus*, Fungus, Zeytinyağı fabrikası atığı, Gibberellik asit (GA_3), Absisik asit (ABA), Zeatin, Bitkisel hormonlar.

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Introduction

Fungi have been reported to synthesize some plant hormones such as gibberellin, abscisic acid (ABA), and cytokinin. GA_3 was found to be synthesized by the fungus *Gibberella fujikuroi (Fusarium moniliforme)* (1-7) as well as by *Agaricus bisporus* (8), *Aspergillus ochraceus, Penicillium funiculosum* (9), *Sphaceloma monihoticola, S. menthae, S. perseae, S. rhois, S. bidentis* (5), *Aspergillus niger* (7), and *Phanerochaete chyrsosporium* ME446 (10). Activities similar to the activity of GA_3 were also detected in *Neurospora crassa* (11). ABA was determined in *Penicillium italicum* (12), *Cercospora rosicola* (13, 14), *Botrytis cinerea* (13, 15, 16), *Cercospora cruenta* (17-19), *Ceratocystis coerulescens* (20), *Cercospora pini-densiflorae* (21), *Polyporus versicolor, Pleurotus ostreatus* (22), *Schizophyllum commune* (23), *Phanerochaete chrysosporium* ME446 (10), and *Pleurotus florida* (24). Cytokinin was found in mycorrhizal fungi (25-27) such as *Taphrina cerasi, T. deformans* (28), *Paxillus involutus, Rhizopogon luteolus* (29), *Fusarium moniliforme, F. culmonum* (30), *Laccaria bicolor, Telephora terestris* (31), *Schizophyllum commune* (23), and *Phanerochaete chrysosporium* ME446 (10).

Using industrial waste as a substrate for extracting GA_3, ABA, and cytokinin from fungi is more economical than using plants. Among industrial wastes, olive oil mill waste causes pollution because of its toxic and antibacterial low-molecular weight phenolic compounds (32). Using olive oil mill waste as a substrate for the production of fungi has dual advantages. First, pollution could be reduced and secondly, the waste could have some economic value in terms of plant hormone production. The goal of this study was the production of plant hormones such as GA_3, ABA, and cytokinin, which have a broad application area in agriculture from olive oil mill waste-feeding fungi such as *Lentinus tigrinus* and *Laetiporus sulphureus*, which have not been used before.

Materials and Methods

In this study, the white-rot fungus *Lentinus tigrinus* and the brown-rot fungus *Laetiporus sulphureus*, which belong to the class Basidiomycetes, were employed to serve the goal of this study. These fungi were a generous gift from the Biology Department of Art and Science Faculty of Inönü University (Malatya -Turkey).

The fungi were grown in a saboraud glucose agar medium in a slanting position. They were kept at 4°C until used. The medium which was diluted by 10% with olive oil mill waste was prepared in 100 ml Erlenmeyer flasks and sterilized at 120°C, at 1 atm for 15 min. Mycelia suspension culture of 2 ml was spread over 100 ml medium. *Lentinus tigrinus* and *Laetiporus sulphureus* were incubated in a static culture at 30°C for 24 hours. Since the olive oil mill waste originates from olive plants, it may contain some plant hormones. In order to clarify fungus produced hormones from those of plant originated hormones, 10%-diluted olive oil mill waste without fungus was used as control medium.

Samples of 100 ml were collected on the 0, 1st, 3rd, 6th, 9th, 12th, 15th, 18th, 21st and 24th days from the incubated fungus culture by filtering extra cellular extracts. Incubation periods included the primary and secondary metabolic phases of the fungus. Each experiment was repeated three times. The extraction, purification and analysis of GA_3, ABA, and zeatin in control experiments, were carried out according to Unyayar et al. (10).

The obtained fungal plant hormones were analyzed by a spectrophotometrical technique (UV-visible spectrophotometer, Shimadzu 2100S) (10). The absorbance of GA_3 extracted from extra cellular suspension cultures of samples and controls were measured at 254 nm. Similarly, ABA and zeatin were analyzed at 263 nm and 269 nm, respectively.

Dry mycelia cultures (mg/100ml) were determined by drying cultures at 65°C for 24 hours and then filtering through Advantec No: 6 paper. The mean and standard error of our data were calculated and comparison was carried out by variance analysis (33). The Duncan test was employed for establishing the significance among the groups.

Results and Discussion

When the total accumulation of GA_3, ABA, and Cytokinin (obtained from controls and sample experiments) and the variations in dry mycelia weights were analyzed together with relevance to the culture period of *Lentinus tigrinus* and *Laetiporus sulphureus* (Tables 1-6); the total amounts of GA_3, ABA, and zeatin separately obtained from the fungal culture was higher than the ones obtained from the control samples on each day of the culture period ($P<0.05$). It was believed that both fungi had completed their primary metabolism and commenced their secondary metabolic phase after 12 days of incubation.

It was found that in *Lentinus tigrinus* culture, the synthesis of three forms of GA_3 occurred on all days but not on the 3rd and 21st days (Table 1). Even though it was considered that GA_3 was synthesized during the primary metabolic phase of the fungus, the majority of the synthesis took place during the secondary metabolic phase (Table 1). In the culture of *Lentinus tigrinus*, the maximum accumulation of total GA_3 occurred on the 18th day of the culture period as 221680.73 µg/ml ($P<0.05$). From Table 2, it was understood that there were some newly synthesized three forms of GA_3 at the 1st hour, and on the 3rd, 12th, and 18th days of the culture period. Moreover, although GA_3 was detected in the secondary metabolic phase, the main synthesis occurred in the primary metabolic phase (Table 2). *Laetiporus sulphureus* produced the maximum amount of total GA_3 on the 3rd day of incubation as 247395.27 µg/ml ($P<0.05$).

In the current study, the production of GA_3 during the secondary metabolic phase of *Lentinus tigrinus* and *Laetiporus sulphureus* was well correlated with previous studies (10, 34, 35). On the other hand, GA_3 synthesis was also observed during the primary metabolic phase of both fungi in this study. Therefore, GA_3 could be considered to be a primary metabolite, which is in accordance with the work of Unyayar et al. (10).

Table 1. The equivalent amounts of free, bound and total GA_3 and dry mycelia weight in *Lentinus tigrinus* culture as a function of culture period (mean of three replicas, and ± standard error).

CULTURE PERIOD (DAY)	EQUIVALENT AMOUNT OF GA_3 (µg/mL)			DMW (mg/100mL)
	FREE	BOUND	TOTAL	
Control	31473.38 ± 174.15	10236.37 ± 391.78	41709.76 ± 234.24	
1ST hour	59453.26 ± 363.36	33441.61 ± 701.25	92894.87 ± 1036.10	4.90 ± 0.21
3	84034.82 ± 2498.48	14889.26 ± 2493.97	98924.08 ± 153.86	11.73 ± 0.49
6	69974.86 ± 2409.81	37460.37 ± 2782.33	107435.23 ± 511.72	29.83 ± 0.26
9	109924.63 ± 1702.81	15939.65 ± 1528.87	125864.27 ± 580.18	45.20 ± 0.49
12	127610.53 ± 815.28	17303.12 ± 477.22	144913.60 ± 367.90	64.13 ± 0.45
15	171690.17± 2549.14	20130.82 ± 188.09	191821.00 ± 2735.90	63.23 ± 0.13
18	197931.40 ± 13783.42	23749.32 ± 3470.82	221680.73 ± 12069.51	57.90 ± 0.12
21	52611.15 ± 371.54	16245.08 ± 302.88	68856.22 ± 672.89	45.63 ± 0.32
24	161801.50 ± 1488.61	25537.71 ± 578.47	187339.20 ± 910.21	43.10 ± 0.20

Table 2. The equivalent amounts of free, bound and total GA_3 and dry mycelia weight in *Laetiporus sulphureus* culture as a function of culture period (mean of three replicas, and ± standard error).

CULTURE PERIOD (DAY)	EQUIVALENT AMOUNT OF GA_3 (µg/mL)			DMW (mg/100mL)
	FREE	BOUND	TOTAL	
Control	31473.38 ± 174.15	10236.37 ± 391.78	41709.76 ± 234.24	
1ST hour	179086.60± 1694.00	36468.94 ± 296.13	215555.53± 1429.24	3.40± 0.32
3	177971.47± 6590.34	69423.83± 10558.23	247395.27± 4347.21	9.60± 0.15
6	55569.10 ± 833.18	28830.64 ± 544.73	84399.73 ± 309.96	20.10± 0.10
9	43184.25 ± 2957.35	14555.41 ± 2156.46	57739.66 ± 1323.09	37.20± 0.15
12	64660.09 ± 772.11	19243.09 ± 170.59	83903.17 ± 616.54	51.03± 0.81
15	47374.92 ± 528.54	36883.81 ± 1200.15	84258.73 ± 691.53	49.87± 0.45
18	67128.10 ± 343.78	27999.39 ± 589.69	95127.48± 575.34	49.67± 0.30
21	59204.43 ± 394.28	36250.11 ± 243.92	95454.54 ± 447.00	44.70± 0.36
24	43712.62 ± 1029.09	24039.24 ± 478.00	67751.86 ± 574.42	42.56± 0.37

It was observed that the synthesis of GA_3 during the secondary metabolic phase of both fungi cultures were coincident with a decrease in food source and suggested a relationship between hormone synthesis and food expenditure. It was suggested that fungi particularly fed on nitrogen among the supplied food. When the secondary metabolic phase was completed, they began producing gibberellic acid (36). Rademacher (36) suggested that in *Gibberella fujikuroi* culture, nitrogen blocked the synthesis of gibberelic acid by means of a reaction between NH_4^+ and 1,2-GA_4-dehydrogenase, which had a vital role in gibberellin synthesis.

Cihangir and Aksoz (7) investigated the efficiency of GA_3 synthesis in 5 different strains of *Aspergillus niger*. Among them, *A. niger* NRRL 567 synthesized 100.29 mg/l, *A. niger* Fursan - 150.35 mg/l, *A. niger* NRRL 328 - 67.50 mg/l, *A. niger* ATTC 2601 - 52.50 mg/l and *A. niger* NRRL 334 - 45.00 mg/l. Some of the researchers reported that the G_5 strain of *Gibberella fujikuroi* produced 120.00 mg/l GA_5 (37). Saucedo et al. (38) determined that 15-day incubation of *Gibberella fujikuroi* yielded 45.00 mg/l GA_3. Kumar and Lansone (39) found that the P-3 strain of *Gibberella fujikuroi* generated 0.58-0.66 mg GA_3/l/hour. In a shaken culture of *Phanerochaete chrysosporium* ME446 strain, Unyayar et al. (10) obtained the total amount of GA_3 as 137.72 µg/ml for the primary metabolic phase on the 9th day and 386.63 µg/ml for the secondary metabolic phase on the 12th day. It was quite obvious from the above references that the *Lentinus tigrinus* and *Laetiporus sulphureus* used in this study had a more efficient mechanism of GA_3 production as compared to other fungi.

It was found that there was newly synthesized ABA in the culture of *Lentinus tigrinus* at the 1st hour, and on the 3rd, 6th, 15th, 18th days of incubation (Table 3). Although ABA synthesis took place during the primary metabolic phase of the fungus, the main synthesis period was the secondary metabolic phase of the fungus (Table 3). The maximum level of total ABA synthesis of *Lentinus tigrinus* was detected as 163.57 µg/ml on the 18th day of the incubation period ($P<0.05$).

Table 3. The equivalent amounts of free, bound and total ABA and dry mycelia weight in *Lentinus tigrinus* culture as a function of culture period (mean of three replicas, and ± standard error).

CULTURE PERIOD (DAY)	EQUIVALENT AMOUNT OF ABA (µg/mL)			DMW (mg/100mL)
	FREE	BOUND	TOTAL	
Control	47.35 ± 1.53	13.50 ± 1.31	60.85 ± 0.80	
1ST hour	43.99 ± 1.49	31.62 ± 2.05	75.61± 0.57	4.90± 0.21
3	64.85 ± 4.29	32.73 ± 2.63	97.58 ± 1.82	11.73± 0.49
6	105.53 ± 4.44	25.90 ± 2.78	131.43 ± 2.26	29.83± 0.26
9	114.84 ± 4.86	18.01 ± 4.38	132.85 ± 1.35	45.20± 0.49
12	77.22 ± 5.61	17.37 ± 3.70	94.59 ± 2.01	64.13± 0.45
15	116.68 ± 3.38	33.24 ± 2.19	149.92 ± 2.94	63.23± 0.13
18	127.19 ± 2.98	36.38 ± 2.39	163.57 ± 1.13	57.90± 0.12
21	81.27 ± 6.70	45.24 ± 1.31	126.51 ± 5.41	45.63± 0.32
24	66.97 ± 5.01	42.13 ± 1.61	109.10 ± 3.73	43.10± 0.20

When the accumulation of ABA was analyzed in the *Laetiporus sulphureus* cultures, it was found that there was newly synthesized ABA at the 1st hour, and on the 15th and 18th days of the incubation period (Table 4). Although ABA synthesis was observed during the secondary metabolic phase of the fungus, the main synthesis period was the primary metabolic phase of

the fungus (Table 4). The maximum level of total ABA synthesis of *Lentinus tigrinus* was detected as 123.96 µg/ml at the 1st hour of the incubation period ($P<0.05$).

Table 4. The equivalent amounts of free, bound and total ABA and dry mycelia weight in *Laetiporus sulphureus* culture as a function of culture period (mean of three replicas, and ± standard error).

CULTURE PERIOD (DAY)	EQUIVALENT AMOUNT OF ABA (µg/mL)			DMW (mg/100mL)
	FREE	BOUND	TOTAL	
Control	47.35 ± 1.53	13.5 ± 1.30	60.85 ± 0.79	
1ST hour	84.82 ± 2.16	39.12 ± 0.02	123.96 ± 2.17	3.40± 0.32
3	65.73 ± 2.82	34.57 ± 4.52	100.30 ± 5.45	9.60± 0.15
6	42.78 ± 5.22	28.19 ± 4.14	70.67 ± 1.71	20.10± 0.10
9	44.16 ± 1.88	20.54 ± 1.29	64.71 ± 0.62	37.20± 0.15
12	49.92 ± 2.23	17.55± 0.39	67.46 ± 1.84	51.03± 0.81
15	51.06 ± 5.11	31.48 ± 3.85	82.54 ± 6.82	49.87± 0.45
18	65.11 ± 3.72	23.04 ± 1.61	88.15 ± 2.58	49.67± 0.30
21	53.11 ± 1.09	24.18 ± 0.59	77.29 ± 0.72	44.70± 0.36
24	45.64 ± 1.56	25.41± 1.78	71.05 ± 3.33	42.56± 0.37

Our data, which indicated the onset and gradually increasing synthesis of ABA at the end of the primary metabolic phase, were also supported by the results of Griffin and Walton (40). They also claimed that in the shaken culture of *Cercospora rosicola*, 27 mg/l ABA was synthesized after 25 days incubation. It was found that high phosphate concentration inhibited ABA accumulation. In the *Cercospora rosicola* culture 6 mg/100ml ABA accumulation was reported after 30 days incubation (20), whereas Kettner and Dorffling (20) found maximum ABA accumulation as 3.5 ng/ml following 30 days incubation for the culture of *Ceratocystis coerulescens*. The maximum ABA accumulation in the shaken culture of *Pleurotus florida* was observed to be 2.78 mg/l after 24 days. However, after 1 day incubation in a static culture (24), the accumulation of ABA was 2.55 mg/l. Yesilada et al. (22) claimed that following 20 day incubation in a static culture, *Polyporus versicolor* synthesized a maximum of 1.39 mg ABA /l and *Pleurotus ostreatus* synthesized maximum 1.28 mg ABA /l. Unyayar et al. (10) reported that there was 13.16 µgABA/ml accumulation following 15 days incubation in a shaken culture of *Phanerochaete chrysosporium* ME 446. *Botrytis cinerea* produced a maximum of 1.60 ng ABA/ml (16). From the above data, it is clear that *Lentinus tigrinus* and *Laetiporus sulphureus* synthesized more ABA than the others employed in this study.

When the accumulation of three forms of zeatin was analyzed in the cultures of *Lentinus tigrinus* and *Laetiporus sulphureus* separetely, it was found that there was newly synthesized zeatin on the 1st hour, and on the 12th and 15th days of the incubation period (Tables 5 and 6). It was considered that maximum zeatin synthesis occurred at the beginning of the primary and

Table 5. The equivalent amounts of free, bound and total zeatin and dry mycelia weight in *Lentinus tigrinus* culture as a function of culture period (mean of three replicas, and ± standard error).

CULTURE PERIOD (DAY)	EQUIVALENT AMOUNT OF ZEATIN (µg/mL)			DMW (mg/100mL)
	FREE	BOUND	TOTAL	
Control	2.72 ± 0.03	0.44 ± 0.03	3.16 ± 0.02	
1ST hour	6.62 ± 0.12	5.42 ± 0.06	12.04 ± 0.06	4.90± 0.21
3	4.68 ± 0.19	3.51 ± 0.26	8.19 ± 0.07	11.73± 0.49
6	6.46 ± 0.60	3.20 ± 0.04	9.66 ± 0.02	29.83± 0.26
9	5.52 ± 0.02	1.35 ± 0.04	6.88 ± 0.05	45.20± 0.49
12	10.27 ± 0.33	3.41± 0.23	13.68 ± 0.11	64.13± 0.45
15	20.97 ± 1.03	2.59 ± 0.17	23.56 ± 0.86	63.23± 0.13
18	14.45 ± 0.18	6.12 ± 0.47	20.57 ± 0.43	57.90± 0.12
21	6.38 ± 0.08	7.45 ± 0.42	13.83 ± 0.38	45.63± 0.32
24	5.40 ± 0.11	1.67 ± 0.15	7.07 ± 0.10	43.10± 0.20

Table 6. The equivalent amounts of free, bound and total zeatin and dry mycelia weight in *Laetiporus sulphureus* culture as a function of culture period (mean of three replicas, and ± standard error).

CULTURE PERIOD (DAY)	EQUIVALENT AMOUNT OF ZEATIN (µg/mL)			DMW (mg/100mL)
	FREE	BOUND	TOTAL	
Control	2.72 ± 0.03	0.44 ± 0.04	3.16 ± 0.02	
1ST hour	5.34 ± 0.05	4.25 ± 0.19	9.59 ± 0.25	3.4 ± 0.32
3	9.84 ± 0.19	0.33 ± 0.19	10.17 ± 0.03	9.6 ± 0.15
6	6.55 ± 0.05	2.72 ± 0.08	9.27 ± 0.02	20.1 ± 0.1
9	3.63 ± 0.15	0.81 ± 0.50	4.44 ± 0.11	37.2 ± 0.15
12	5.39 ± 0.71	4.99 ± 0.70	10.38 ± 0.12	51.03 ± 0.81
15	4.68 ± 0.35	14.05 ± 0.29	18.73 ± 0.07	49.87 ± 0.45
18	8.44 ± 0.16	2.98 ± 0.13	11.42 ± 0.07	49.67 ± 0.3
21	3.81 ± 0.20	0.44 ± 0.13	4.25 ± 0.07	44.7 ± 0.36
24	1.86 ± 0.09	1.53 ± 0.27	3.39 ± 0.17	42.56 ± 0.37

secondary metabolic phases of the fungi (Tables 5 and 6). The maximum accumulation of total zeatin in the culture of *Lentinus tigrinus* and *Laetiporus sulphureus* was found to be 23.56 µg/ml and 18.73 µg/ml respectively on the 15th day of the incubation period ($P<0.05$).

Johnston and Trione (28) researched the kinetin equivalence of cytokinin produced in *Taphrina deformans* and *Taphrina cerasi* culture. They reported that *T. deformans* synthesized 4 µg/ml cytokinin while *T. cerasi* synthesized 2 µg/ml cytokinin (28). Unyayar et al. (10) claimed that in a shaken culture of *Phanerochaete chrysosporium* ME 446, the total zeatin amount was

detected as 10.23 µg/ml at the 1st hour and the maximum total zeatin was obtained on the 12th day as 10.31 µg/ml. These results supported our notion that maximum zeatin synthesis took place at the beginning of the primary and the secondary metabolic phases of *Lentinus tigrinus* and *Laetiporus sulphureus*. Based on the above data, *Lentinus tigrinus* and *Laetiporus sulphureus*, used in this study, were claimed to synthesize more zeatin.

As a result of the current study, olive oil mill waste was shown to be a suitable substrate for *Lentinus tigrinus* and *Laetiporus sulphureus* in order to produce bound and/or free GA_3, ABA, and zeatin from either their primary metabolic phase or secondary metabolic phase. Furthermore, this was the first time that *Lentinus tigrinus* and *Laetiporus sulphureus* were employed for the production of plant hormones.

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