Bangladesh J. Bot. **46**(3): 1099-1104, 2017 (September) Supplementary

DEGRADATION OF BENZOIC ACID IN ROOT EXUDATES OF FLUE-CURED TOBACCO BY *ASPERGILLUS FLAVUS* Z5

XIN LI AND QIANG LI*

Hunan Agricultural University, Changsha 410128, China

Keywords: Root exudates, Allelochemical, Benzoic acid, *Aspergillus flavus*, Degradation

Abstract

The allelochemical benzoic acid, released from plant roots, is an important inhibitor of plant growth. It was found that *Aspergillus flavus Z5* isolated from flue-cured tobacco root reduced the damage of tobacco seedlings caused by root exudates. Results indicated that *A. flavus* had a positive effect on root morphological characteristics, chlorophyll content, and photosynthetic characteristics. *A. flavus* grown on Martun medium with benzoic acid as the sole carbon source in a wide range of concentrations. The optimum growth concentration for this strain was 800 μmol/l, and the highest degradation rate was 2.833 mg/d between 7 and 9 days of fungal culture.

Introduction

Allelopathic compounds, released by plants or microorganisms, can both promote or inhibit the growth of other organisms (Rice 1984). Plants like tobacco, rice, wheat, pea, pepper, and eggplant release allelochemicals mainly through their roots (Hao *et al.* 2010, Yu *et al.* 2000). Plant root exudates contain large amounts of phenolics, including nicotine, salicylic acid, benzoic acid, 2-hydroxypropanoic acid, scopoletin, coumarin, p-hydroxybenzoic acid, vanillic acid, 3-hydroxy-hydrocinnamic acid and phenylacetic acid, resulting in the inhibition of plant growth (Bertin *et al.* 2003, Inderjit *et al.* 2008, Lannucci *et al.* 2013). Therefore, the reduction of allelochemicals in plant root exudates has become an important research topic.

In the previous studies carried out by the authors, one strain of *Aspergillus flavus* was isolated from root exudates of flue-cured tobacco (*Nicotiana tabacum* L.) using Martun medium with benzoic acid as the sole carbon source. Present research was undertaken to investigate how this strain promoted the growth of tobacco seedlings and degraded benzoic acid.

Materials and Methods

The strain *Aspergillus flavus* Z5 (KT633952) was isolated from root exudates of flue-cured tobacco variety K326 in Guizhou Province, China. The isolate was maintained on Martun medium (glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4$, $7H_2O$ 0.5 g, 1/300 Rose Bengal solution 3 ml), and Martun medium with benzoic acid was used as the sole carbon source.

Root exudates were extracted at Fuquan test base Guizhou Academy of Tobacco Science 90 days after transplantation. Tobacco roots were washed and cultured in sterile water in aerated cultures (electromagnetic air pump, 80W) for 48 hrs with changing tobaccos six times continuously. The root exudates were collected filtering the sterile water with sterile pledgets, and then stored at room temperature.

Fifteen-day old seedlings were selected as the test materials in greenhouse at Guizhou Academy of Tobacco Science. Consistently growing tobacco plants were picked up for experiments. Fifty ml of different solutions were poured in seedlings tray everyday for 45 days

*Author for correspondence: <zqiangli@126.com>.

(Table 1). Twelve seedlings were planted for each treatment and each treatment was repeated for three times. Morphological characteristics of root treatment, photosynthetic characteristics, and chlorophyll content were measured after 60 days.

Table 1. Different treatments in seedlings tray for tobacco seedlings.

Treatment	Solution
M1	50 ml water
M2	50 ml root exudates
M3	5 ml strain suspension and 45 ml root exudates

Plant root system Win RHIZO was used to determine total root surface area, branching number, average root diameter, root tip number, and total root length per unit square meter. Portable photosynthesis measuring instrument Li-6400XT was used in the determination of photosynthetic characteristics from 8 a.m. to 4 p.m. in sunny days. The measurement indexes included net photosynthetic rate (Pn), stomatal conductance (Cond), transpiration rate (Tr) and the concentration of CO_2 between cell gap (Ci). Ultraviolet spectrophotometer TU-1901 was used to measure chlorophyll content. Five millimeter mycelia were placed into liquid Martun medium with benzoic acid as the sole carbon source. Benzoic acid with a concentration of 0, 400, 800, 1200, 1600 and 2000 µmol/l was used in concentration screening tests. After culture in a shaker at 28ºC and 170 rpm for 15 days, the mycelia were filtered and moved to the thermostatic drying drum wind (Model:DGG-9246, Shanghai Qi Yan Scientific Instrument Co., Ltd.) at the temperature of 60ºC for two hours. The weight of dry mycelia was measured by electronic balance (Model: PL303, Mettler Toledo Instrument Co., Ltd.).

The concentration of benzoic acid in the medium was measured by GC-MS every 3 days (Yan *et al.* 2009). Each measurement was repeated three times. Eight hundred µmol/l benzoic acid was chosen for the analysis of the rate of degradation of benzoic acid by *A. flavus* Z5. The degradation rates were calculated in each time period.

All data were processed with the SIGMASTAT Statistical Software Package (SPSS Science, Chicago).

Results and Discussion

Flue-cured tobacco seedlings treated with water (M1) had better root morphological characteristics than those treated with root exudates (M2) or a mixed solution of root exudate and *A. flavus* suspension (M3). At the same time, total root surface area, units of square meters of soil total root length, the number of branches; the root average diameter and the apical number were higher in seedlings treated with M3 than in those treated with M2, with an improvement of 5.84, 6.90, 8.15, 9.43 and 6.65%, respectively. The results indicated that root exudates inhibited the growth of tobacco seedling roots, while *A. flavus* suspension reduced the damage from root exudates. (Table 2).

Seedlings treated with water had the highest content of chlorophyll a, chlorophyll b, carotenoid, and total chlorophyll, that were 0.35, 0.19, 0.071 and 0.61 mg/g, respectively. In seedlings treated with M3, the content of chlorophyll a, chlorophyll b, carotenoid and total chlorophyll were 0.28, 0.16, 0.053 and 0.49 mg/g, respectively. However, when compared to seedlings treated M2, M3-treated seedlings had an increase of 7.69, 14.29, 15.22 and 16.67% in the content of chlorophyll a, chlorophyll b, carotenoid and total chlorophyll, respectively. These

results indicated that strain suspension could decrease the effects of root exudates on chlorophyll content (Table 3).

The results of determination of photosynthetic characteristics followed a trend similar to that of root morphological characteristics and chlorophyll content. Seedling treated with water displayed the highest indexes of photosynthetic characteristics, with net photosynthetic rate (Pn), stomatal conductance (Cond), transpiration rate (Tr), and CO_2 concentration between cell gap (Ci) of 13.2, 0.47 and 6.4 mmol/(m^2/s) and 376.9 µmol/mol, respectively. M3 treated seedlings had an increase in Pn, Cond, Tr and Ci of 5.71, 13.79, 8.33 and 5.82% compared to that of root exudates only, respectively (Table 4).

Table 2. Effects of different treatments on tobacco seedlings roots.

Treatment	Total root surface area cm²	Units of square meters of soil total root length (cm/mm³)	Number of branches	Root av. diameter mm	Apical number
M1	54.5a	285.3a	1395.5a	0.69a	369.2a
M2	34.1c	226.1b	1033.0b	0.53b	287.3b
M3	39.5b	241.7b	1117.2ab	0.58ab	306.4ab

Different letters in the same column mean significantly difference at 0.05 level (the same below).

Table 3. Effects of different treatments on tobacco seedlings chlorophyll content.

Treatment	Chlorophyll a content (mg/g)	Chlorophyll b content (mg/g)	Carotenoid content (mg/g)	Total chlorophyll content (mg/g)
M1	0.35a	0.19a	0.071a	0.61a
M2	0.26b	0.14b	0.046b	0.42b
M3	0.28b	0.16ab	0.053ab	0.49b

Table 4. Effects of different treatments on tobacco seedlings photosynthetic characteristics.

Treatment	Pn [µmol·(m^2/s)]	Cond [µmol·(m^{-2}/s)]	Tr [mmol·(m^{-2}/s)]	Ci (µmol· mol^{-1})
M1	13.2a	0.47a	6.4a	376.9a
M2	10.5c	0.29b	4.8b	297.4b
M3	11.1b	0.33ab	5.2b	314.7ab

The biological dry weights of *A. flavus* under different concentrations of benzoic acid varied significantly (Fig. 1). When treated with 800 µmol/l benzoic acid, the dry weight of *A. flavus* was 0.765 g. With a concentration of 400 µmol/l, the dry weight of *A. flavus* was 0.282 g. However, in cultures with 1200, 1600 and 2000 µmol/l, the dry weights showed more significant difference from 400 and 800 µmol/l, with the weights being 0.063, 0.033 and 0.063 g, respectively. The results indicated that 800 µmol/l was the optimum concentration of benzoic acid for *A. flavus* Z5 growth.

The content of benzoic acid showed a significant difference in test concentrations. When treated at the third day, the differences were not significant. However, the degradation diverged with the cultivation time. We measured the biggest difference at day 15 (Fig. 2). We observed that culture with 800 µmol/l benzoic acid had the most significant effect on depredation amount of benzoic acid, with the amount reaching 19.5 mg at the 15th day. Meanwhile, in cultures with 400 µmol/l benzoic acid, the degradation of benzoic acid was significantly lower, yielding only a residual amount of benzoic acid of 7.5 mg. However, benzoic acid degradation did not significantly change with concentrations of 1200, 1600 or 2000 µmol/l in the growth medium, where the residual amounts of benzoic acid were 0.45, 0.27 and 0.45 mg, respectively.

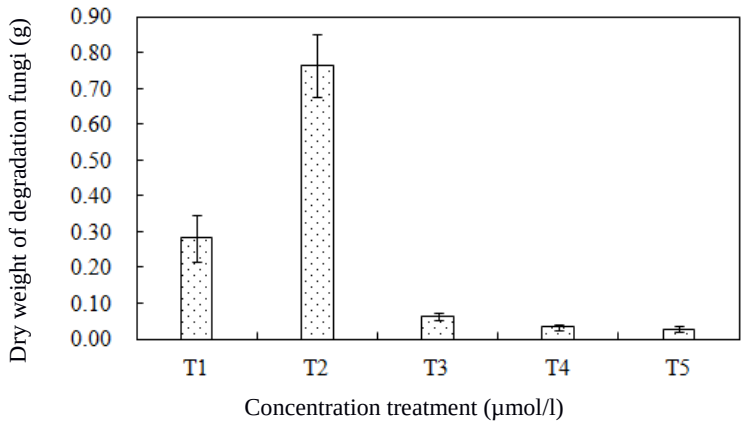

Fig. 1. Dry weights of *A. flavus* with different concentrations of benzoic acid. (T1, T2, T3, T4 and T5 represent 400, 800, 1200, 1600 and 2000 µmol/l, respectively.).

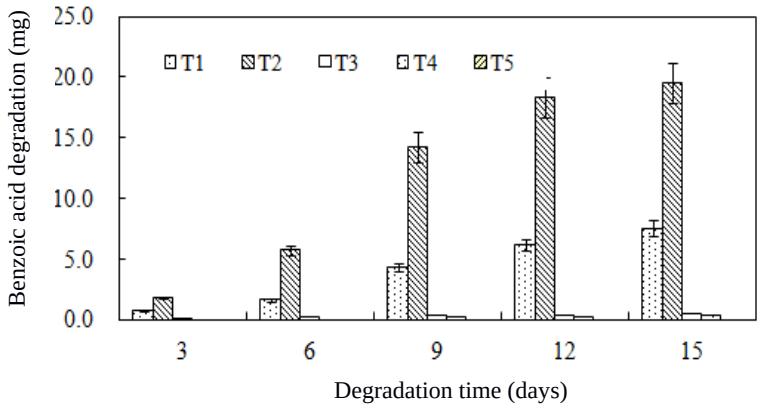

Fig. 2. Degradation amounts of different concentrations of benzoic acid by *A. flavus*.

The degradation rate of benzoic acid declined with the increase of culture time (Fig. 3). The residual amount of benzoic acid was 1.8 mg between day 0 and 3, 3.9 mg between day 4 and 6, 8.5 mg between day 7 and 9, 4.1 mg between day 10 and 12, and 1.2 mg between day 13 and 15, respectively. The degradation rate was 0.600, 1.300, 2.833, 1.367 and 0.400 mg/d, respectively.

Using Martun medium with benzoic acid as the sole carbon source, we isolated one *A. flavus* strain. *A. flavus* Z5 promoted root growth, chlorophyll content, and photosynthetic characteristics in tobacco seedlings when compared to seedling treated with root exudates only. Results indicated that *A. flavus* Z5 reduced the inhibitory effects of root exudates on the growth of tobacco seedlings.

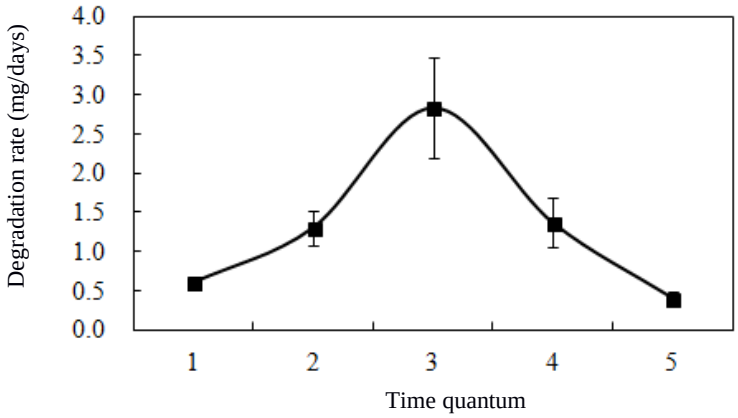

Fig. 3. Degradation rate of 800 µmol/l benzoic acid with *A. flavus* at different time periods (1, 2, 3, 4, and 5 represent 0 to 3rd, 4 to 6th, 7 to 9th, 10 to 12th and 13 to 15th days, respectively.)

Benzoic acid is the main compound in tobacco root exudates to inhibit tobacco growth (Gao *et al.* 2012). In our study, *A. flavus* Z5 degraded benzoic acid to promote tobacco seedlings growth. *A. flavus* Z5 could grow on Martun medium with benzoic acid as the sole carbon source at different concentrations, with 800 µmol/l being the best concentration for its growth. We found that *A. flavus* Z5 had high capacity to degrade benzoic acid, with the highest degradation rate of 2.833 mg/d between 7 and 9 days of the fungal culture. Previous studies reported on other microorganisms were able to degrade benzoic acid (Wackett 2007, Denef *et al.* 2004, Cao *et al.* 2008, Karlsson *et al.* 2004). Here, we found that the *A. flavus* Z5 degraded benzoic acid. It may have used the same degradation mechanism as *Aspergillus niger* (Boschloo *et al.* 1990).

Here we described the ability of *A. flavus* Z5 to degrade benzoic acid, providing a new tool to control root exudates in the growth of tobacco. However, we studied only the influence of *A. flavus* on the growth of tobacco seedlings. Therefore, further studies are needed to investigate the influence of this strain on the growth of tobacco in the field.

Acknowledgments

The authors gratefully acknowledge the funding and support from Hunan Province scientific research innovation project (CX2015B238).

References

Bertin C, Yang X and Weston LA 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil **256**: 67-83.

Boschloo JG, Pafen A, Koot T, Tweel WJJVD, Gorcom RFMV and Cordewener JHG 1990. Genetic analysis of benzoate metabolism in *Aspergillus niger*. Appl. Microbiol. Biot. **34**(2): 225-228.

Cao B, Geng A and Loh KC 2008. Induction of *ortho*- and *meta*-cleavage pathways in *Pseudomonas* in biodegradation of high benzoate concentration: MS identification of catabolic enzymes. Appl. Microbiol. Biot. **81**(1): 99-107.

Denef VJ, Park J, Tsoi TV, Rouillard JM, Zhang H, Wibbenmeyer JA, Verstraete W, Gulari E, Hashsham SA and Tiedje JM 2004. Biphenyl and benzoate metabolism in a genomic context: outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl. Environ. Microb. **70**(8): 4961-4970.

Gao XX, Yu HY, Zhang JG, Liu S, Shi P and Wang S 2012. Identification of chemical compositions of Root exudates from flue-cured tobacco and their influence to seed germination. Chinese Tobacco Science **33**(3): 87-91.

Hao W, Ren L and Ran W 2010. Alleopathic effects of root exudates from watermelon and rice plants on *Fusarium oxysporum* f. sp. *niveum*. Plant Soil **336**: 485-497.

InderjitSeastedt TR, Callaway RM, Pollock JL and Kaur J 2008.Allelopathy and plant invasions: traditional, congeneric, and bio-geographical approaches. Biol. Invasions **10**: 875-890.

Karlsson A, Beharry ZM, Matthew Eby D, Coulter ED, Neidle EL, Kurtz DM Jr, Eklund H and Ramaswamy S 2002. X-ray crystal structure of benzoate 1, 2-dioxygenase reductase from *Acinetobacter* sp. strain ADP1. J. Mol.Biol. **318**(2): 261-272.

Lannucci A, Fragasso M, Platani C and Papa R 2013. Plant growth and phenolic compounds in the rhizosphere soil of wild oat (*Avena fatua* L.). Plant Science **4**: 1-7.

Rice E L 1984. Allelopathy. New York: Academic Press Inc.

Wackett LP 2003. *Pseudomonas putida* - A versatile biocatalyst. Nat. Biotechnol. **21**(2): 136-138.

Yu J Q, Shou S Y, Qian Y R, Zhu Z J and Hu W H. 2000. Autotoxic potential of cucurbit crops. Plant Soil, **223**: 147-151.

Yan Z, Zhang X, Bing W, Zhao D, Mei L and Cui Y 2009. Degradation of benzo (a) pyrene in Yangtze River source water with functional strains. Ecotoxicology **18**(6): 742-747.

(Manuscript received on 31 March, 2017; revised on 22 September, 2017)