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ORIGINAL PAPER

Control of lethal browning of tissue culture plantlets of Cavendish banana cv. Formosana with ascorbic acid

W. H. Ko · C. C. Su · C. L. Chen · C. P. Chao

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Abstract Cavendish banana cv. Formosana is a high yielding commercial cultivar resistant to race 4 of *Fusarium oxysporum* f. sp. *cubense*. Mass micropropagation of this cultivar has a serious problem of high mortality due to lethal browning of plantlets. The mineral contents in leaves and corms of diseased and healthy plantlets were similar. Amendment of culture medium with anion exchange resins, cation exchange resins, polyvinylpyrrolidone or activated charcoal did not reduce the disease incidence. However, addition of ascorbic acid to the surface of culture medium not only prevented the development of lethal browning but also greatly increased the number of plantlets produced. Even at 0.005% ascorbic acid was able to reduce the disease incidence by more than 60% and caused over 8-fold increase in number of plantlets produced. When cultures raised from 12 different Formosana corms were tested, ascorbic acid was able to reduce disease incidence by an average of 83%, and increase the number of plantlets in each test. When diseased plantlets were transferred to culture medium with ascorbic acid, all of them recovered, and resumed normal growth and multiplication, while all control plantlets on culture medium without ascorbic acid died after one month.

Keywords Lethal browning · Cavendish banana · Ascorbic acid

W. H. Ko (✉)
Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan
e-mail: kowh@dragon.nchu.edu.tw

C. C. Su · C. L. Chen · C. P. Chao
Taiwan Banana Research Institute, Chiuju, Pingtung, Taiwan

Abbreviation
SM Smith and Murashige

Introduction

Fusarium wilt of Cavendish banana (*Musa acuminata* Colla, AAA) caused by race 4 of *Fusarium oxysporum* Schlecht: Fr. f. sp. *cubense* (E. F. Smith) Snyder & Hansen is a major threat to banana production in Taiwan, Canary Islands, Malaysia, and some parts of Australia and South Africa (Hwang and Ko 2004). From 1970s to 1980s, it was the most devastating disease of banana in Taiwan affecting more than 1,000 ha and killing about half a million trees each year (Su et al. 1986).

In 1984, a project was initiated at the Taiwan Banana Research Institute to screen the Cavendish plantlets from tissue culture for resistance to Fusarium wilt. Six resistant clones were obtained within 2 years. A commercially acceptable resistant clone was registered as cv. Tai Chiao No.1 for commercial planting in 1992 (Hwang and Ko 2004). From 1992 to 2001, 1.6–3.0 million Tai Chiao No.1 plantlets were planted each year on 800–1,500 ha of infested orchards, representing about 75% of total infested orchards in Taiwan. In 2002, a new resistant clone with good eating quality and exceptionally high yield was registered as cv. Formosana. Since then, more than 2 million plantlets of this new cultivar were produced for commercial planting (Hwang and Ko 2004).

The major problem in mass micropropagation of cv. Formosana plantlets has been the high mortality rate due to lethal browning in the third generation of tissue culture. In a severe case, all the plantlets in the flask died because of such ailment. Similar phenomenon has been observed in tissue cultures of other banana cultivars and other plant species (Martin et al. 2007). In the process of testing

various methods for alleviation of the disorders, ascorbic acid was found to be capable of preventing the lethal browning of plantlets. The compound also restored health to diseased plantlets. Details of the study are reported herein.

Materials and methods

Preparation of tissue culture

Meristem-tip cultures of Cavendish banana cv. Formosana were derived from shoot apices following the method of Ma and Shii (1972). Decapitated shoot apices of suckers were surface sterilized by brief dipping in 70% ethanol and removal of outer layers under aseptically conditions. Surface sterilized explants (ca 10 × 10 × 6 mm^3) were incubated at 25°C under continuous cool-white fluorescent light on the medium of Smith and Murashige (1970) (SM medium) which contains Murashige and Skoog (1962) salt mixture plus (per liter) 0.4 mg thiamine–HCl, 100 mg L-tyrosine, 100 mg myoinositol, 11 μM indole-3-acetic acid, 22 μM benzyladenine, 160 mg adenine sulphate, 30 g sucrose, and 7 g agar. The medium was adjusted to 5.8 with 2 N KOH and 50 ml was dispensed per culture vessel (6 cm diameter, 10 cm high).

After incubation on the above medium for one month, about 5–10 new buds emerged from each explant. Tissues with new buds were cut into smaller pieces each with 1–2 buds and the divided tissues were transferred to fresh medium for formation of second generation buds. After growth and multiplication for one month, tissues were divided again and transferred to SM medium with various treatments.

Tissue analysis

To determine if lethal browning is caused by deficiency or toxicity of mineral nutrients, the mineral contents of leaves and corms of diseased and healthy plantlets of Cavendish banana cv. Formosana were analyzed by the Physiology Laboratory of Taiwan Banana Research Institute. Tissues of leaves and corms were washed, oven-dried, ground into powder, and treated with concentrated sulfuric acid in a heating digester. Nitrogen and phosphate were analyzed calorimetrically with an UV-1 spectrophotometer (Thermo Electron Corporation, Waltham, MA), while potassium was analyzed by Ependorf flame photometer (Ependorf, Hamburg, Germany) (Richards 1993). Calcium, magnesium, iron, manganese, copper and zinc were analyzed by atomic absorption spectrophotometer (Perkin Elmer Optoelectronics, Fremont, CA) after tissue powder was ashed and dissolved in 3N HCl (Osborne and Voogt 1978).

Addition of adsorptive or antioxidant material to medium

To study the effect of adsorptive materials on the incidence of lethal browning of banana tissue culture plantlets, the SM medium was amended with 10% each of Dowex anion exchange resins (Dow Chemical Co., Midland, MI), Lowatit cation exchange resins (Bayer Corporation, Pittsburgh), polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, Mo), or activated charcoal (Taipei Chemical Industry Co., Hsinchu, Taiwan). To remove possible inhibitory substances, 100 g each of this compound was soaked in 1,000 ml distilled water. The pH of mixture was adjusted to 5.7 and filtered through Whatman no. 1 filter paper after shaking for 30 min (Ho et al. 2007). To study the effect of antioxidant on the incidence of lethal browning of banana tissue culture plantlets, 10 ml of 0.01% ascorbic acid solution was added to the surface of SM medium in each flask after autoclaving. The pH of ascorbic acid solution was adjusted to 5.7 and sterilized by filtration through 0.22 μm Millex-GV membrane filter (Millipore Co., Bedford, MA). Five flasks were used for each treatment and five buds on a piece of banana tissue were placed in each flask. The experiment was repeated twice.

To determine the effect of ascorbic acid concentration on the incidence of lethal browning and the plantlet propagation of third generation plantlets of cv. Formosana, 10 ml of ascorbic acid solution at 0, 0.005%, 0.01% or 0.02% was added to the surface of SM medium in a flask. Three flasks were used for each concentration, and five buds on a piece of banana tissue were placed in each flask. The experiment was repeated twice.

To determine if ascorbic acid can be used as a general treatment for reducing the incidence of lethal browning and increasing the number of plantlets produced, SM medium with 0.01% ascorbic acid added onto the surface was used to grow the plantlets of third generation of cv. Formosana derived from 12 corms obtained from different banana trees on March 14 and May1, 2007 at the experimental farm of Taiwan Banana Research Institute.

Results

Tissue analysis showed that the mineral contents of leaves were similar to those of corms with the exceptions of Fe and Zn which were about two fold higher in corms than leaves (Table 1). The mineral contents in diseased leaves were similar to those in healthy leaves, and the same is true for diseased corms and healthy corms (Table 1).

The adsorptive materials including anion exchange resin, cation exchange resin, polyvinylpyrrolidone and activated charcoal were not effective in reducing the

Table 1 Mineral contents of leaves and corms from diseased and healthy tissue culture plantlets of Cavendish banana cv. Formosana

Source of sample	% (Dry weight basis)					µg/g			
	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Leaves									
Diseased plantlets	6.2	0.2	16.7	0.7	0.2	217.4	261.3	13.2	10.6
Healthy plantlets	6.8	0.2	17.1	0.7	0.2	216.8	240.2	10.4	9.8
Corms									
Diseased plantlets	5.3	0.2	6.4	0.6	0.3	464.1	216.7	14.8	20.4
Healthy plantlets	5.1	0.2	6.3	0.8	0.3	484.6	268.0	12.8	18.8

Table 2 Effect of adsorptive or antioxidant material on the incidence of lethal browning of tissue culture plantlets of Cavendish banana cv. Formosana

Treatment	Concentration (%)	No./flask	
		Diseased plantlets[a]	Healthy plantlets
Anion exchange resin	10	3.3 A[b]	6.3 B
Cation exchange resin	10	4.7 A	4.0 BC
Polyvinylpyrrolidone	10	4.3 A	4.7 BC
Activated charcoal	10	3.3 A	2.0 C
Ascorbic acid	0.01	0.0 B	20.3 A
Control		3.7 A	5.7 BC

[a] Plantlets with brown leaves

[b] Means in each column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference tests

incidence of lethal browning (Table 2). However, an antioxidant tested was found to be very effective in reducing the lethal browning of cv. Formosana plantlets. The number of diseased plantlets per flask was reduced from 3.7 in the control to 0 on SM medium covered with 0.01% ascorbic acid (Table 2; Fig. 1). Ascorbic acid also increased the number of healthy plantlets per flask from 5.7 in the control to 20.3.

Results showed that even at the concentration of 0.005%, ascorbic acid was able to reduce the number of diseased plantlets per flask from 10.7 without ascorbic acid to 4.0, and increase the number of healthy plantlets per flask from 1.7 in the control to 15.0 (Table 3). Ascorbic acid at the concentrations of 0.01% and 0.02% reduced the number to diseased plantlets per flask to 0, and increased the number of healthy plantlets per flask to 20.3 and 18.0, respectively.

Plantlets originating from different Formosana corms were different in sensitivity to lethal browning. Although 88% plantlets derived from corm no. 4 were diseased, only 11% from corm no. 5 showed lethal browning symptoms. The result showed that ascorbic acid was effective in reducing the incidence of lethal browning of cv. Formosana plantlets derived from all the 12 corms tested (Table 4). The amount of disease reduction ranged from 41% in plantlets from corm no. 4 to 100% in plantlets from corm nos. 5 and 10, with an average disease reduction rate of 83%. Addition of ascorbic acid to SM medium also

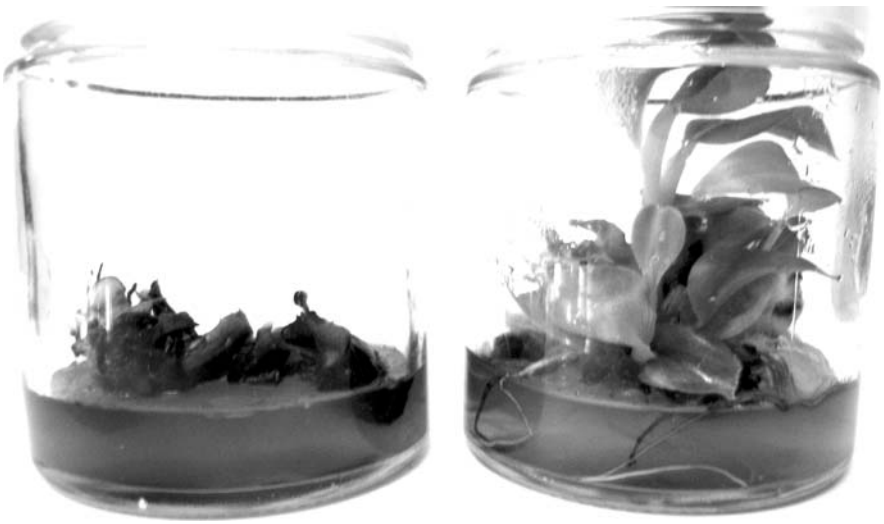

Fig. 1 Plantlets of Cavendish banana cv. Formosana growing on Smith and Murashige medium covered with 0% (left) or 0.01% (right) ascorbic acid

Table 3 Effect of ascorbic acid concentration on the incidence of lethal browning and the plantlet propagation potential of tissue culture of Cavendish banana cv. Formosana

Concentration (%)	No./flask	
	Diseased plantlets[a]	Healthy plantlets
0	10.7 A[b]	1.7 B
0.005	4.0 B	15.0 A
0.01	0.0 C	20.3 A
0.02	0.0 C	18.0 A

[a] Plantlets with brown leaves

[b] Means in each column followed by the same letter are not significantly different ($P = 0.05$) according Fisher's least significant difference tests

increased the number of healthy plantlets ranging from 9.3 to 24.3 produced in each flask for all the corms tested (Table 4).

To determine if plantlets suffering from lethal browning can recover from the ailment by transfer to SM medium with 0.01% ascorbic acid added to the surface, third generation cv. Formosana tissues were cut into pieces each containing five plantlets with brown leaves. The divided tissues were each placed in a flask containing SM medium with or without ascorbic acid. Five flasks were used for each treatment. After one month on SM medium with ascorbic acid, all the diseased plantlets recovered and resumed normal growth and multiplication (Table 5). All the diseased plantlets died on SM medium without ascorbic acid after one month.

Discussion

This study showed that lethal browning of Cavendish cv. Formosana plantlets was not due to deficiency or toxicity of mineral nutrients. Banana tissues are known to contain large amount of latex and phenolic compounds (Wu and Su 1990). Certain banana tissue cultures may cause darkening of medium due to exudation and oxidation of phenolic compounds resulting in the formation of quinones which are highly reactive and toxic to plant tissue (Titov et al. 2006). However, accumulation of toxic substances in the medium apparently was not the cause of lethal browning of cv. Formosana plantlets because amendment of culture medium with adsorptive materials did not alleviate the problem (Table 2). Among the adsorptive materials tested in this study, activated charcoal and polyvinylpyrrolidone are able to adsorb phenol-like substances (Pierik 1987), and anion and cation exchange resins are known to adsorb ionic toxic substances (Ko and Hora 1972).

The effectiveness of ascorbic acid in resolving the lethal browning problem of cv. Formosana plantlets suggests that

Table 4 Effect of ascorbic acid on the incidence of lethal browning and the propagation potential of tissue culture plantlets originated from corms of Cavendish banana cv. Formosana obtained from different mother plants

Corm no.	Treatment	No./flask	
		Diseased plantlets[a]	Healthy plantlets
1	Ascorbic acid	1.0*	15.3*
	Control	8.6	3.8
2	Ascorbic acid	4.5	9.3*
	Control	9.8	3.5
3	Ascorbic acid	1.4*	13.4*
	Control	8.2	3.6
4	Ascorbic acid	8.6	10.3*
	Control	14.6	2.0
5	Ascorbic acid	0.0	16.5
	Control	2.0	15.5
6	Ascorbic acid	0.3	19.8*
	Control	3.8	10.3
7	Ascorbic acid	0.4*	22.0*
	Control	4.8	8.2
8	Ascorbic acid	1.0	17.8
	Control	3.8	12.4
9	Ascorbic acid	0.3	21.0*
	Control	3.0	10.8
10	Ascorbic acid	0.0	24.3*
	Control	1.5	14.0
11	Ascorbic acid	0.3*	22.5*
	Control	3.5	9.8
12	Ascorbic acid	0.3*	23.5*
	Control	2.8	10.0

[a] Plantlets with brown leaves

[b] Means in each column for each corm no. followed by * are significantly different from the control ($P = 0.05$) according to Student's *t*-tests

such disorder may be caused by oxidation of phenolic compounds inside the young plantlet leaves. It is conceivable that ascorbic acid may have been absorbed by the plantlets, translocated to leaves, and prevented the oxidation of phenolic compounds on the target site. Further studies are needed to verify the hypothesis. It is not known if the problem of lethal browning is related to resistance of Formosana cultivar to race 4 of *F. oxysporum* f. sp. *cubense*. Phenolic compounds have been shown to be involved in resistance of some host plants to pathogens (Nicholson and Hammerschmidt 1992).

Our results showed that ascorbic acid not only can prevent the occurrence of lethal browning in subsequently produced plantlets, it can also stop the progress of browning in affected plantlets. When diseased plantlets were transferred to SM medium containing ascorbic acid,

Table 5 Recovery of Cavendish banana cv. Formosana plantlets suffering from lethal browning after 1 month on SM medium with or without ascorbic acid

Treatment	Plantlets recovered (%)[a]	No./flask	
		Diseased plantlets	Healthy plantlets
Ascorbic acid	100	1.0 ± 0.8[b]	23.3 ± 2.4
Control	0	5.0 ± 0.0[c]	0.0 ± 0.0

[a] A total of 25 diseased plantlets tested

[b] Plantlets with mild leaf browning

[c] All plantlets died

leaf browning ceased to progress and the leaves produced thereafter did not develop browning symptoms (Table 5).

Plantlets originated from different corms of Cavendish cv. Formosana displayed considerable variation in susceptibility to lethal browning. Plantlets derived from corm nos. 2 and 4 were highly susceptible, while those from corms nos. 5 and 10 were highly resistant (Table 4). This could be due to inherent variation of this cultivar in susceptibility to lethal browning. It could also be the result of somaclonal variation which is an important characteristic of banana tissue culture (Hwang and Ko 1986). Table 4 showed different ability of corms from different plants to produce plantlets under the influence of ascorbic acid. The information is important to the estimation of the number of corms required for obtaining sufficient numbers of plantlets needed by growers each year.

Ascorbic acid is known to decay rapidly in plant tissue culture media (Elmore et al. 1990). When 0.05% ascorbic acid was added to MS medium before autoclaving, it was not effective in controlling the lethal browning (unpublished data). The approach of adding ascorbic acid onto the surface of the medium reported here should also be useful for alleviating similar problem occurring during the micropropagation of other banana cultivars and other plant species (Martin et al. 2007).

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