

Full Length Research Paper

In vitro* culture method of powdery mildew (*Oidium heveae* Steinmann) of *Hevea brasiliensis

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Accepted 8 May, 2012

A method for culturing powdery mildew (*Oidium heveae*) from isolated leaves of *Hevea brasiliensis* was evaluated, which included three steps: Leaves and fungi selection, nutrient solution and culture dish preparation, fungi inoculation and culture. The culture time and produced conidia number were considered as decision index. We tested the influence of micro components of nutrient solution including 6-benzylaminopurine (6-BA), salicylic acid (SA) and vitamin C (VC) and evaluated the culture difference of various leaf phenological phases and rubber tree clones. The results show that the longest culture time of isolated leaves emerged on modified Murashige and Skoog (MS) macro elements with 4 mg/L 6-BA, 20 mg/L SA, 1 mg/L VC. The colour phase leaf was the preferable choice for culturing average 15 to 16 days and producing 3.2222×10^6 mL⁻¹ conidia. The culture effects of using various rubber clones were different and higher resistance clones cultured less conidia. The method leading to mass production of powdery mildew was simple using a climate incubator to resolve problems linked to season and space limitation and preservation of powdery mildew. This method could improve rubber resistance breeding process.

Key words: *Hevea brasiliensis*, *Oidium heveae*, *in vitro* culture, nutrient solution, phenological phase.

INTRODUCTION

Powdery mildew of rubber tree (*Oidium heveae*) is an important leaf disease of *Hevea brasiliensis* plantations worldwide (Beeley, 1933; Saranya et al., 2005). As an obligatory parasite, *O. heveae* requires living host cells from fresh rubber leaves, buds, inflorescences and other young tissues for growth (Saranya et al., 2005; Huang 2005). However, in powdery mildew resistance breeding as well as in studies of biological and physiological characteristics and virulence differentiation of the pathogen, large amounts of powdery mildew as infected material are needed. In the literature, three main methods

have been reported for *in vitro* culturing and preserving of powdery mildew from other plants: (1) benzimidazole used as protective green agent, inoculating powdery mildew conidia on leaves for *in vitro* culture (He et al., 1998; Miazzi et al., 1997); (2) sexual generation cleistothecium of powdery mildew used to inoculate fresh leaves (Dahmen et al., 1983); (3) powdery mildew isolates maintained by inoculating conidia on plantlet leaves or cotyledons (Li et al., 1985; Seifi et al., 2012). Unfortunately, these methods are not feasible in *O. heveae*, because benzimidazole is not effective as green agent for rubber tree leaves; *O. heveae* has not been found sexual stage. In addition, the production of rubber trees plantlet is still at experimental stage, and cannot yet provide large number of plantlets for the culture and preservation of *O. heveae*. Therefore, the objective of our research is to establish a kind of simple and efficient *in vitro* culture method of *O. heveae*. This new method will allow the production of the mass culture of *O. heveae*,

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Abbreviations: 6-BA, 6-Benzylaminopurine; SA, salicylic acid; VC, vitamin C; ST, sodium thiosulfate; AC, activated carbon; MS, Murashige and Skoog; NS, nutrient solution.



Figure 1. In vitro culture of powdery mildew (*Oidium heveae*) of *Hevea brasiliensis*. a, three kinds of phenological phase leaves of bronze, Colour and light green of GT1; b, fresh powdery mildew asexual conidia; c, NS7 nutrient solution; d, Colour phase leaves with nutrient solution in culture dish; e, *in vitro* culture in climate incubator; f, Colour phase leaves cultured for 9 day of GT1.

consequently aid in its research and improve rubber resistance breeding process. In this study, the influences of 6-benzylaminopurine (6-BA), salicylic acid (SA) and vitamin C (VC) of nutrient solution for *in vitro* culture were investigated by orthogonal experimental design.

MATERIALS AND METHODS

Plant and fungus materials

Bronze phase, colour phase and light green phase, three kinds of phenological phase leaves of *H. brasiliensis* clones GT1 were used for culture. Daily, 27 leaves (including three repeats) belonging to each of the three phases were observed the conidia number, and recorded culture time. In addition, colour phase leaves of two other *H. brasiliensis* clones RRIC52 and PB5/51 were used for comparison. Usually, clone RRIC52 was the resistant check and PB5/51 was the susceptible check in rubber tree powdery mildew resistance identification (Huang, 2005). These materials were provided by the Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences (Figure 1a).

Fresh powdery mildew conidia (*O. heveae*) were collected from infected leaves of seedling clone GT1 from rubber tree germplasm nursery of Rubber Research Institute and used within 24 h as inoculums (Figure 1b).

Nutrient solution and cultural dish preparation

The nutrient solutions were prepared with different concentrations of 6-BA (0, 4, 8 and 12 mg/l), SA (0, 10, 20 and 30 mg/l) and VC (0,

0.2, 0.5 and 1 mg/l) (Table 1) and invariant concentration of modified MS large elements (0.4 g/L ST + 1 g/L AC + 1/5 MS macro elements: 3.3 g/L ammonium nitrate, 3.8 g/L potassium nitrate, 0.88 g/L calcium chloride, 0.74 g/L magnesium sulfate, 0.34 g/L potassium dihydrogen phosphate). The pH of the nutrient solution was adjusted to 6.0 and all media were autoclaved for 20 min at 121°C (Figure 1c).

The cultural dish included white porcelain dish, sand and polymerase chain reaction (PCR) plate. Clean sand was put in the white porcelain dish with sterile water to keep cool and humid, PCR plate was placed in the sand. The nutrient solution was transferred in the PCR plate hole using pipette gun, while the control group using sterile water.

Rubber leaves from each of the three phenological phases of GT1 were washed with sterile water three times, soaked in 0.5% sodium hypochlorite solution for 5 minutes, and then washed with sterile water three times again. After draining water, the leaves were inserted in PCR plate holes containing nutrient solution or sterile water (Figure 1d). The numbers of days until leaves wilting were recorded.

Fungi inoculation, culture and microscopy observation

Fresh powdery mildew asexual conidia from susceptible rubber leaves of GT1 were brush in sterile water for conidial suspension preparation and the concentration was adjusted to 1×10^6 conidia per mL. The conidial suspension was then brushed on the rubber tree leaves (Wang et al., 2010) and leaves were placed in a climate incubator for culture till wilt under 23°C, 90% humidity, 16 h light to 8 h dark (Plant protection Research Institute, 1982; Liyanage et al., 1987) (Figure 1e).

To observe and analyze the conidia growth effectually, the culture

Table 1. Comparison analysis of the orthogonal experiment for leaf culture time of *H. brasiliensis* clone GT1.

Nutrient solution treatment	Micro component (mg/L) ^a			Culture time (day) ^{* e}			
	6-BA ^b	SA ^c	VC ^d	Bronze phase	Colour phase	Light green colour phase	Duncan grouping ^f colour
NS1	0	0	0	5.18 ± 0.30	9.29 ± 0.07	13.40 ± 0.26	E
NS 2	0	10	0.2	6.96 ± 0.12	10.26 ± 0.18	15.96 ± 0.39	CD
NS 3	0	20	0.5	7.26 ± 0.56	13.67 ± 0.35	17.89 ± 0.25	B
NS 4	0	30	1	5.96 ± 0.37	10.29 ± 0.61	16.74 ± 0.84	CD
NS 5	4	0	0.2	6.74 ± 0.12	10.89 ± 0.14	17.51 ± 0.04	CBD
NS 6	4	10	0	7.03 ± 0.48	12.14 ± 0.09	17.14 ± 0.46	CB
NS 7	4	20	1	8.22 ± 0.31	15.78 ± 0.38	20.37 ± 0.18	A
NS 8	4	30	0.5	6.48 ± 0.53	12.44 ± 0.34	18.37 ± 0.36	CB
NS 9	8	0	0.5	6.11 ± 0.46	11.29 ± 0.29	15.62 ± 0.73	CD
NS 10	8	10	1	6.92 ± 0.36	13.48 ± 0.34	16.37 ± 0.24	CB
NS 11	8	20	0	7.19 ± 0.22	12.45 ± 0.75	18.11 ± 0.59	CB
NS 12	8	30	0.2	6.26 ± 0.34	11.59 ± 0.07	17.4 ± 0.43	CBD
NS 13	12	0	1	6.37 ± 0.38	11.33 ± 0.24	17.74 ± 0.14	CBD
NS 14	12	10	0.5	6.52 ± 0.19	12.48 ± 0.94	16.56 ± 0.27	CBD
NS 15	12	20	0.2	5.89 ± 0.77	10.37 ± 0.27	18.29 ± 0.08	CBD
NS 16	12	30	0	6.33 ± 0.69	9.18 ± 0.36	15.22 ± 0.37	ED
				1.64 ^{ab} (P = 0.2777)	4.94 ^{*ab} (P = 0.0464)	6.31 ^{*ab} (P = 0.0276)	
				2.70 ^{ac} (P = 0.1388)	6.29 ^{*ac} (P = 0.0278)	8.65 ^{*ac} (P = 0.0134)	
				0.44 ^{ad} (P = 0.7328)	5.70 ^{*ad} (P = 0.0343)	4.02 ^{ad} (P = 0.0695)	

*Significantly different at P = 0.05; a, variance analysis with SAS program; b, F-value of 6-BA; c, F-value of SA; d, F-value of VC; e, Culture time: the culture number of days from leaf inoculation to leaf wilted; f, Duncan multiple range test.

leaves was cut in square of 2 cm × 2 cm to eliminate the influence of leaf size, and washed with 1mL sterile water. Conidia were observed and counted using blood counting chamber by microscope. The max conidia number was the maximum value of a series of conidia numbers during culture time.

Comparison *in vitro* culture using various rubber tree clones

Colour phase leaves of three *H. brasiliensis* clones GT1, RRIC52 and PB5/51 were used to analyse the culture difference. The optimal nutrient solution was chosen: 4

mg/L 6-BA, 20 mg/L SA, 1 mg/L VC and modified MS large elements. Fungi inoculation, culture and observation methods were same as above.

Statistical analysis

In this study, the orthogonal design of nutrient solution L₁₆ (4⁵) was used and treatments were repeated three times. The data were analyzed by means of variance analysis (ANOVA) on the statistical package of SAS program (Version 9.0). The leaves culture time were analyzed by Duncan's multiple range tests (P = 0.05) to evaluate the effects of different concentrations of 6-BA, SA and VC.

RESULTS

Determination of nutrient solution for screening

To evaluate the effects of nutrient solution components on rubber tree leaves, the first series of experiments was performed on GT1 clone at three phenological phases. Sixteen nutrient solution treatments based on modified MS large elements with different concentrations of 6-BA, SA and VC were tested using an orthogonal design.

Table 2. The variance analysis of powdery mildew *in vitro* culture on various phenological stages leaves of *H. brasiliensis* clone GT1.

Phenological phase ^a	Culture time (day) ^c		Max conidia number (1×10^6) ^b	
	Sterile water	NS7 Nutrient solution	Sterile water	NS7 Nutrient solution
Bronze	3.14 ± 0.30	8.22 ± 0.31	0.6925 ± 0.0444	1.2667 ± 0.0038
Colour	5.29 ± 0.07	15.78 ± 0.38	0.7333 ± 0.0148	3.2222 ± 0.1852
Light green	7.40 ± 0.26	20.37 ± 0.18	0.8026 ± 0.0122	1.7778 ± 0.1111
	31009.6 ^a (P < 0.0001)		1810.21 ^a (P < 0.0001)	
	2822.5 ^b (P < 0.0001)		156.69 ^b (P < 0.0001)	
	536.18 ^{ab} (P < 0.0001)		541.45 ^{ab} (P < 0.0001)	

a, F-value of phenological phase; b, F-value of culture components; ab, F-value of factor interaction of phenological phase and culture components; c, Culture time: the culture number of days from leaf inoculation to leaf wilted.

The nutrient solution NS7 gave the best results since the number of days until wilting of the leaves was significantly higher than with the other 15 nutrient solutions. NS7 represented the level of treatment: 4 mg/L 6-BA, 20 mg/L SA, 1 mg/L VC (Table 1). Significant differences for culture time of three phenological periods were tested by variance analysis among micro components of (p = 0.05), SA (p = 0.05) and VC (p = 0.05). At the bottom of the Table 1, ANOVA analyses indicated that three micro components 6-BA, SA and VC had no significant effect on the culture time of bronze leaves with F-values of 1.64, 2.70 and 0.44 respectively. On the contrary, these micro components had significant effect on culture time of rubber leaves at colour phase and light green phase. The most powerful factor affecting the culture time of bronze leaves was SA (F = 6.29), followed by VC (F = 5.70) and 6-BA (F = 4.94). In addition, 6-BA and SA increased significantly the culture time of leaves at light green phase. The effect of SA (F = 8.65) was stronger than the effect of 6-BA (F = 6.31) (Table1).

Comparison of culture effect of various phenological stages leaves

The first series of experiments showed that NS7 nutrient solution allowed the longest culture time for GT1 leaves. The production of conidia was then in NS7 nutrient solution. Three phenological phases were tested. Two factor interaction significance tests were conducted to analyze the influence of GT1 leaf phenology phase (bronze, dis colouration, light green) and culture medium (NS7 nutrient solution, sterile water) on culture time and max conidia number of powdery mildew (Table 2). The results showed that culture medium had a significant effect on culture time and maximum conidia number (P < 0.0001). The culture time and max conidia number of three phenological period leaves enhanced when GT1

leaves were cultured in NS7 nutrition solution compared to sterile water (Table 2). Leaf phenology phase also had a significant effect on culture time and max conidia number (P < 0.0001). The highest max conidia number was obtained at the colour phase, although the longest culture time was obtained when leaves were at the light green phase (Table 2). In view of the interaction of phenological period and culture medium, the optimal nutrient solution was NS7 and the most suitable leaf phenology phase was colour phase components had significant effect culture time of rubber leaves at colour phase and

Comparison of culture effect of various rubber tree clones

To analyze the culture difference of various rubber tree clones, colour phase leaves of clones GT1, PB5/51 RRIC52 were tested for powdery mildew *in vitro* culture. The optimal nutrient solution NS7 was used for these experiments. The results indicate that the conidia numbers observed on the three clones presented the same change trend during whole culture period. Culture days 1-6 were slow-growth period of conidia number, culture days 7-8 were rapid-growth period, and culture days 9-14 were steady-high-growth period (Figure 2). At the same time, culture time of clone PB5/51 was 14 days; culture time of clones GT1 and RRIC52 was longer for 16 days and conidia numbers were slightly reduced in last two days. The average conidia number during 9-14 culture day of three clones were 2.9168×10^6 for GT1, 3.0946×10^6 for PB5/51, 2.4590×10^6 for RRIC52. Therefore, NS7 nutrient solution and the colour phase leaf can isolate culture powdery mildew of various clones effectively.

DISCUSSION

To solve the problem of *in vitro* culture of powdery mildew

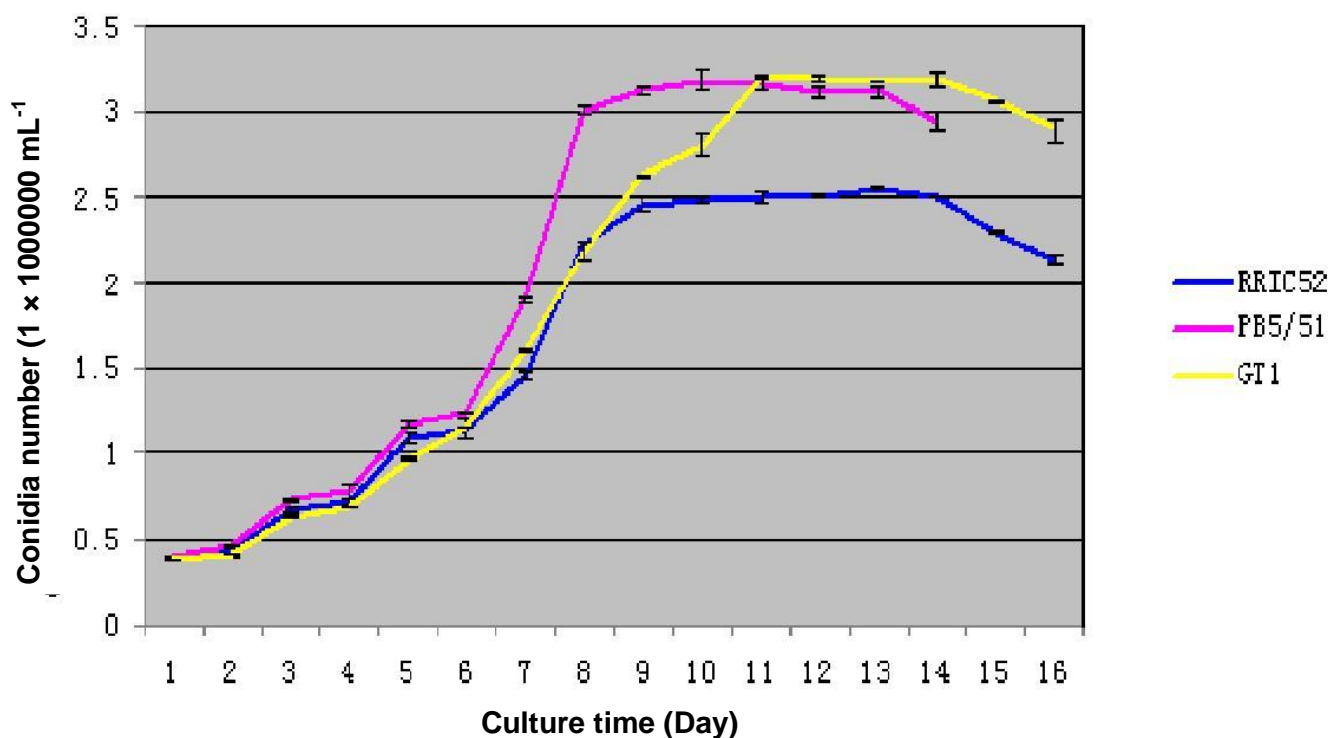


Figure 2. Conidia number change of three rubber clones on colour phase leaves

of rubber tree, this study proposes an effective nutrient solution including macro and micro components. Macro component was modified MS large elements (1/5 MS macro elements + 0.4 g/L ST + 1 g/L activated charcoal (AC), which provided basic nutrients and metabolic environment for ST was beneficial for delaying leaf senescence as ethylene inhibitors, and AC was good for adsorption of harmful substances and inhibition of ethylene production (Zhou et al., 2010; Murashige and Skoog, 1962, Bhat and Chandel, 1991; Chen, 2011). Three kinds of micro components were 6-BA, VC and SA. The 6-BA and VC assisted eliminating free radicals and delaying leaf senescence (Hua et al., 2010). In the study, SA had significant correlation with culture time of colour leaf and green leaf than 6-BA and VC. One explanation could be that SA was useful in inhibiting bacterial reproduction and reducing catheter blockage (Chen et al., 1993; Sticher et al., 1997; Yuan et al., 1994; Zheng et al., 2006). In summary, these components played a very important role in prolonging the culture time of rubber tree leaves.

Culture time and conidia number were the key factors to influence and evaluate *in vitro* culture effect of the different media. In 16 nutrient solution treatments, Light green leaf of GT1 had the longest average culture time (13.4 to 20.37 day), which was higher than colour leaf (9.29 to 15.78 day) and much higher than bronze leaf (5.18-8.22 day). But the max conidia number of light

green leaf (1.7778×10^6) was lower than the colour leaf (3.2222×10^6), which possibly was related to the powdery mildew infection mechanisms. When powdery mildew conidia germinate, it forms a tube and penetrates through the plant's cuticle using mechanical pressure and the action of enzymes. The light green leaf is a stabilized leaf, presenting a thick cuticle and therefore imposing additional barriers for powdery mildew infection and reproduction (Shan et al., 1999; Zhang et al., 2010). Therefore, the colour phase leaf was the preferable choice for *in vitro* culture of powdery mildew.

The study also found that the conidia number produced by the use of different colour phase leaves belonging to various rubber clones were different. Our results of the average conidia number during 9 to 14 culture day of three clones were PB5/51 (3.0946×10^6) > GT1 (2.9168×10^6) > RRIC52 (2.4590×10^6), which were consistent with the field resistance identification results of PB5 / 51 (disease index, DI = 53.1) > GT1 (DI = 29.4) > RRIC52 (DI = 20) by Yu et al (1992). The usual resistance identification method of powdery mildew (*O. heveae*) is field identification, which includes spraying inoculums of *O. heveae* on the rubber clone's bronze-coloured leaves during suitable climate and carrying resistance identification in the spring after leaves aging. The disadvantage is need longer time to wait leaf aging, and may present incomplete symptom for environment influence (Wastie, 1975). Compared with the

conventional method, the condition of *in vitro* culture is easy to control; the tree cut leaves can still grow normally. Furthermore, resistance identification and virulence evaluation of powdery mildew will not be affected by seasonal restrictions improving work efficiency. If the correlation between conidia number and field resistance of various rubber tree clones can be further analyzed, a new way of rapid resistance evaluation of rubber tree to *O. heveae* may bring to effect.

ACKNOWLEDGEMENTS

This work was supported by the Fundamental Research Funds for Rubber Research Institute, CATAS (1630022011003) and the Crop Germplasm Resources Protection Project of Chinese Agricultural Ministry (10RZZY-06).

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