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利用辣椒疫霉培养滤液体外筛选胡椒抗瘟病无性系研究

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摘要：在胡椒（*Piper nigrum* Linn.）茎尖丛生增殖技术的基础上，以印尼大叶种“Lampong Type”无菌实生苗作外植体源，利用辣椒疫霉(*Phytophthora capsici*)培养滤液对胡椒茎尖及其增殖形成的丛生芽进行体外选择。辣椒疫霉培养滤液的不同灭菌方法对辣椒疫霉培养滤液的毒性影响显著，过滤灭菌方式可以保持辣椒疫霉培养滤液的毒性，而高温高压灭菌方式则不能。随着辣椒疫霉培养滤液浓度的增加，茎尖和丛生芽的存活率和增殖率都在下降。在存活的茎尖或丛生芽培养中，一部分可正常增殖，其余的形成愈伤组织，或者保持生长停滞的休眠状态。在选择性培养基上继代培养 2 次后进行生根和移栽，利用离体叶片针刺接种法对温室条件下生长的移栽植株进行抗瘟病测定。以 3 次抗病检测均无明显症状的植株作为抗病株。随着辣椒疫霉培养滤液浓度的增加，得到的再生植株数量降低，但其中抗病株的比例提高。利用过滤灭菌方式加入选择性培养基的处理中，25%、50%和 75%的辣椒疫霉培养滤液分别获得 1 株、4 株和 3 株抗病株，分别占各处理再生植株总数的 1.54%、20.00%和 42.86%，共获得 8 株，占该组处理再生植株总数的 8.70%。

关键词：胡椒；胡椒瘟病（根腐病）；辣椒疫霉；培养滤液；体外选择；体细胞无性系变异；抗病性
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In vitro Selection of Black Pepper (*Piper nigrum* Linn.) Somaclones Resistant to Foot Rot Using Culture Filtrate of *Phytophthora capsici*

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Abstract: Based on shoot-tip multiplication technique, the application of *in vitro* selection of black pepper somaclones resistant to foot rot disease caused by fungus *Phytophthora capsici* was carried out using a large-leaf variety Daye (Lampong Type) which is widely cultivated in Hainan but highly susceptible to *P. capsici* as explant resources. The results demonstrated that sterilization methods significantly influenced on the toxicity of the culture filtrate of *P. capsici*. The fungal filtrate toxicity could be maintained using filtrate sterilization by addition of the fungal filtrate into selective medium instead of autoclave sterilization. The survival rate of shoot tips and multiple shoots decreased with the increasing concentrations of the fungal culture filtrate. Some cultures blackened and died finally, and some formed calli or remained dormant. After 2 subcultures onto the same selective medium, microshoots were rooted *in vitro* and transferred to the greenhouse conditions for screening the resistance to fungus *P. capsici*. The plants exhibited no external symptoms of the disease during three successive times of resistance assay once two weeks were considered to be resistant. As the concentrations of fungal culture filtrate increased, the total number of regenerated plants obtained declined but the frequencies of plants resistant to *P. capsici* increased. 1 (1.54%), 4 (20.00%) and 3 (42.86%) disease resistant somaclonal variants were obtained at concentration of 25%, 50% and 75% fungal culture filtrate as selective agents, respectively, with the total number of 8 plants resistant to *P. capsici*.

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Key words: Black pepper; *Piper nigrum*; Foot rot; *Phytophthora capsici*; Culture filtrate; *In vitro* selection; Somaclonal variation; Disease resistance

— Black pepper (*Piper nigrum* Linn.) is an important tropical spice crop. Whether in cultivation area, yield, or economic value, black pepper has been the leader among spices, and known as "king of the spices" or "black gold". Major countries in production and supply of black pepper include India, Vietnam, Indonesia, Malaysia, Thailand, Brazil, Sri Lanka and China[1].

Foot rot disease (*Phytophthora* foot rot, quick wilt, or sudden-death) is the most serious and dreaded disease affecting black pepper which is caused by the soil borne fungus *Phytophthora capsici* (formerly known as *Phytophthora palmivora* var. *piperis*, or *Phytophthora palmivora* (Butl.) Butler MF4). This disease is prevalent in all pepper growing tracts of the world. This "black pepper killer" is one of the major constraints in black pepper production[2]. Therefore, improvement of crop resistant to foot rot disease has been becoming a major objective of black pepper breeding.

Two essential prerequisites for plant breeding are the presence of sufficient genetic variation and the availability of efficient selection procedures[3]. There is no real germplasm which is resistant to foot rot found in *P. nigrum*. Although *P. colubrinum* is reported to be resistant to fungal pathogen *P. capsici* and can serve as potential donor of various resistance traits in black pepper improvement programme[4], but conventional hybridization and grafting of *P. colubrinum* and *P. nigrum* have not been successful as yet due to graft as well as sexual incompatibility[5]. Asymmetric hybridization and genetic transformation for the transfer of quick wilt resistance from *P. colubrinum* to *P. nigrum* is not practical due to lack of the efficient *in vitro* regeneration systems and disease-resistance genes cloned[6]. Somaclonal variation and *in vitro* selection techniques have already been used for obtaining potentially disease-resistant plants of various crops, and some cultivars have been released that derived from somaclonal variation [3,7-9]. Therefore, application of *in vitro* selection of black pepper somaclones resistant to the foot rot may be an attractive alternative.

In the present paper we describe the *in vitro* selection of black pepper somaclones resistant to the foot rot based on the *in vitro* shoot-tip multiplication technique reported by Liu & Zheng[10]. To our knowledge, there have been no reports of use of this system to produce disease-resistant black pepper plants.

1 Materials and Methods

1.1 Plant materials and culture conditions

Shoot tips and multiple shoot buds, initiated from shoot tips excised from 50-day-old aseptic seedlings of a black pepper variety, Daye (Lampong Type), extensively cultivated in China but highly susceptible to *P. capsici*, were used as plant materials for selection. Initiation medium (IM) and multiplication medium (MM) were MS medium + 1.0 mg L^{-1} BA + 0.1 mg L^{-1} IAA + 3% sucrose + 0.75% agar. All cultures were incubated at 26 ± 2℃ under continuous illumination of 56 μ mol $m^{-2}s^{-1}$ cool-white fluorescent lights.

1.2 Preparation of culture filtrate media and selection

The isolate of *P. capsici*, kindly supplied by Plant Protection Institute, Chinese Academy of Tropical Agricultural Science, was cultured on carrot agar medium. To obtain filtrate, cultures were grown in flasks containing 200 ml of liquid carrot medium agitated at 100 rpm for 2 weeks at 25℃. The suspensions were filtered to remove the conidia and mycelia and sequentially filtered through filter paper (Φ60×045). The undiluted culture filtrate was used in preparing toxic media. Toxic media were prepared by substituting fungal culture filtrate for 0% (0 ml L^{-1}), 25% (250 ml L^{-1}), 50% (500 ml L^{-1}), and 75% (750 ml L^{-1}) of the distilled water in the IM or MM described above. The toxic media were autoclaved for 20 min at 121℃ before or after addition of the fungal culture filtrate through 0.2 μm aseptic NALGENE™ millipore membrane. Shoot tips excised from aseptic

seedlings were grown on the selective media for 4 weeks, and surviving shoots and multiple bud clusters from each bottle were pooled and transferred every 4 weeks into the same fresh selective media for 8 weeks. After 12 weeks of culture, the number of shoot tips survived, forming callus, being dormant and forming new shoots were measured. After selection, shoots were rooted in 1/2 MS + 1.0 mg L^{-1} IBA + 0.5 mg L^{-1} IAA + 3% sucrose + 0.75% agar. After 4 weeks of culture, the plantlets were established in pots containing a mix of coir dust, sand and soil (in the proportion of 1:1:1) and were later transferred to the greenhouse.

1.3 Evaluation of regenerated plants for resistance to *P. capsici*

Screening of regenerated plants for resistance to *P. capsici* was conducted using a modified method of Kueh & Khew[11] and Lin & Pan[12]. Two weeks after *in vitro* inoculation of zoospore suspension into wounds, created by puncturing, in three full expanded leaves, the reaction of 3 leaves of individual regenerants to *P. capsici* was observed and the percentage of infected leaf tissue was converted to a graded series: 0 (no lesion on leaf), 1 (appearance of lesion less than 25% leaf areas), 2 (25%–50%), 3 (50%–75%), and 4 (75%–100%). An average disease severity index (ADSI) was calculated from these scores according to the formula: ADSI = [Σ (score × number of leaves corresponding to each score)] /(3×4)×100%. The disease response of the individual regenerants was therefore calculated and classified into 4 groups: resistant (R, 0%, and this means that the plants exhibited no external symptoms of the disease during three successive times of resistance assay once two weeks were considered to be resistant), moderately resistant (MR, <30%), moderately susceptible (MS, 30%–60%), and susceptible (S, 60%–100%).

2 Results

The results demonstrated that sterilization methods had a significantly influence on the toxicity of the culture filtrate of *P. capsici* (Table 1). The percentages of shoot tips survived after selection ranged from 93.33% to 81.42% with the autoclave sterilization of the culture filtrate, and from 70.15% to 30.56% with the filter sterilization. It showed that the fungal filtrate toxicity could be maintained using filtrate sterilization instead of autoclave sterilization. In addition, The percentage of survived shoot tips and multiple shoots and newly initiated shoots decreased with the increasing concentrations of the fungal culture filtrate using the same sterilization method. Of all shoot tips survived, some formed new shoots, others profusely formed callus or remained dormant. The number of shoots produced from treated shoot tips was generally low compared to the controls.

After 2 subcultures onto the same selective medium, microshoots were rooted *in vitro* and transferred to the greenhouse conditions and screened for resistance to *P. capsici*. The plants which exhibited no external symptoms of the disease during three successive times of resistance assay once two weeks were considered to be resistant. No resistant plant was produced with autoclave-sterilized fungal culture

Table 1 Effects of concentrations of culture filtrate of *P. capsici* and sterilization methods on growth of shoot tips of black pepper

Concentrations (%,v/v) of fungal culture filtrate	Number (percentage) of shoot tips					Total shoots obtained
	Surviving	Initiating callus	Dormant	Forming shoots	Shoot tips treated	
Autoclave sterilization						
25	56 (93.33)	2 (3.33)	3 (5.00)	51 (85.00)	60	93
50	54 (83.03)	2 (3.08)	4 (6.15)	48 (73.85)	65	79
75	57 (81.42)	3 (4.29)	4 (5.71)	50 (71.43)	70	77
Filter sterilization						
25	47 (70.15)	6 (8.96)	8 (11.94)	33 (49.25)	67	65
50	32 (50.07)	11 (17.46)	12 (19.05)	9 (14.29)	63	20
75	22 (30.56)	7 (9.72)	11 (15.27)	4 (5.56)	72	7
Control	65 (100.00)	0 (0.00)	1 (1.54)	64 (8.46)	65	175

filtrate. As the concentrations of fungal culture filtrate increased, the total number of regenerated plants obtained declined but the frequencies of plants resistant to *P. capsici* increased. 1 (1.54%), 4 (20.00%) and 3 (42.86%) disease resistant somaclonal variants were obtained at concentration of 25%, 50% and 75% filter-sterilized fungal culture filtrate as selective agents, respectively, with the total number of 8 plants resistant to *P. capsici* (Table 2).

Eight somaclones with the resistance to *P. capsici* were obtained through *in vitro* selection with the fungal culture filtrate of *P. capsici*. In contrast to those, there was no one plant having disease resistance found in the unselected control plants and source plants. Morphological variations such as leaf color change (turned to yellow-green), leaf size change (became small), aberrant leaf laminae, dwarfism and bushiness in apices were also observed *ex vitro*.

3 Discussion

Two prerequisites for a successful *in vitro* selection system with pathogen culture filtrate containing toxins are that toxins which play an important role in pathogenesis or disease development, and that the resistance is expressed at cellular level as well as the whole plant level[3, 7, 9, 13]. The work of Lee [14] demonstrated that toxin inoculation of *Phytophthora palmivora* (i.e. *Phytophthora capsici*) was as effective as fungal inoculation on black pepper cultivars, *Piper colubrinum* and *P. sarmentosum* for the screening of black pepper for foot rot resistance. Our results showed that *in vitro* selection with the filter-sterilized fungal culture filtrate of *P. capsici* for foot rot resistance is effective and inhibition of shoot tip growth and multiplication with the selective medium containing 50% fungal culture filtrate is most suitable. Although the inhibition of the shoot tip growth and proliferation increased with the concentrations of the fungal culture filtrate, but the number and the percentage of shoot tips and multiple shoots survived and of newly initiated shoots dramatically decreased. Only 7 shoots were produced from 22 survived shoot tips and 3 somaclones were screened to be resistant to *Phytophthora capsici* when treated with 75% fungal culture filtrate.

Toxic pathogen culture filtrates and purified toxins have been used for *in vitro* selection and regeneration of disease-resistant plants [15-24]. In consideration of sensitivity of plant cultures to phytotoxins, protoplasts, cell suspension, and callus were used as the selection material units in most experiments. But, one prerequisite of a successful *in vitro* selection system is the establishment of a plant regeneration system. Since there were only several reports on the successful regeneration of black pepper [10, 25-27] and black pepper lacked an efficient and reliable system of plant regeneration from the undifferentiated cultures, the use of an organized culture system such as shoot tip and multiple shoot for *in vitro* selection may be an attractive way, because it may reduce or eliminate some of the problems

Table 2 Reaction of somaclones obtained with different treatments of selection to *P. capsici*

Concentrations of Fungal culture filtrate (%, v/v)	Number and percentage of plants at each resistant level				Number of plants screened
	R	MR	MS	S	
Autoclave sterilization					
25	0 (0.00)	3 (3.23)	37 (39.78)	53 (56.99)	93
50	0 (0.00)	8 (10.13)	27 (34.18)	44 (55.70)	79
75	0 (0.00)	6 (7.79)	27 (35.06)	44 (57.14)	77
Filter sterilization					
25	1 (1.54)	11 (16.92)	28 (43.08)	26 (40.00)	65
50	4 (20.00)	10 (50.00)	5 (25.00)	5 (25.00)	20
75	3 (42.86)	5 (71.43)	2 (28.57)	0 (0.00)	7
Unselected control	0 (0.00)	5 (2.86)	28 (16.97)	140 (84.85)	175
Source plants	0 (0.00)	0 (0.00)	4 (20.00)	16 (80.00)	20

associated with undifferentiated culture systems such as the loss of morphogenetic potential[13, 28]. This study showed that *in vitro* selection of black pepper somaclones resistant to the foot rot based on the shoot-tip proliferation may be feasible, and further studies of the somaclones are needed to understand the stability and inheritance of the disease resistance.

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