A STUDY ON INTERSPECIFIC HYBRIDIZATION BETWEEN

*Piper nigrum* AND *Piper colubrinum*

Chen Yi Shang

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A STUDY ON INTERSPECIFIC HYBRIDIZATION BETWEEN
Piper nigrum AND Piper Colubrinum

CHEN YI SHANG

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DECLARATION

I hereby declare that no portion of the work referred to this thesis has been submitted in support of an application for another degree or qualification to this or any other university or institution of higher learning.

(Chen Yi Shang)

Date: 01st July 2011
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ABSTRACT

Interspecific hybridization between *P. nigrum* L. and *P. colubrinum* Link. was attempted aiming to achieve *Phytophthora* foot rot resistant hybrid. *P. nigrum*, an important spice crop is tetraploid (2n=52) while *P. colubrinum*, a *Phytophthora* disease resistant species of *Piper* is diploid in nature. The main objective of this study is to find out the cross compatibility between the two species. Various experiments were conducted in this study i.e. reproductive biology study on parent plant, study on possible occurrence of apomixes in *P. nigrum*, artificial and natural pollination study and induction of tetraploid *P. colubrinum*. This study eventually proved that interspecific hybridization between *P. nigrum* L. and *P. colubrinum* Link, was incompatible by judging on negative result gathered from morphological and cytological studies on putative interspecific hybrid in first experiment of interspecific hybridization and zero success fruit set in second experiment of interspecific cross (after modified and improved methods of artificial pollination). There was also no germination of pollen of *P. colubrinum* on the stigmatic surfaces of *P. nigrum* in investigation of *in vivo* germination. Before carrying out experiment of interspecific hybridization, the studies were focused on developing a reliable method of artificial pollination between two species of *Piper*. Reproductive biology study on both parent plants i.e. *P. nigrum* (male) and *P. colubrinum* (female) enlightened that artificial pollination was ideally to be carried out approximately starting from 11 a.m until 5 p.m. In pollen viability study of *P. colubrinum*, anther dehiscence was discovered to occur around 9 a.m. and pollen was scientifically proved most viable at two hours after dehiscence and onward. Whilst, stigma receptivity study on *P. nigrum* suggested that stigma at stage 1 i.e. first emergence of stigmata and stage 3 i.e. complete emergence and wide spreading of stigmata were preferably to be
selected for the hybridization. At the same time, apomixes was proved do not contribute to fruit set of *P. nigrum*. Besides reproductive biology study on both parent plants to assist artificial pollination, reliable methods of eliminating self pollination were also developed. A reliable method of eliminating self pollination can be achieved by sampled of single flower only per inflorescence together with bagging of inflorescence and physical emasculation. The applicability of these methods was proved practical. Eventually, chromosome doubling of *P. colubrinum* through colchicine was carried out as one of the possible solution for the cross incompatibility between *P. nigrum* and *P. colubrinum*. However, preliminary results showed the treatment was not successful. None of the colchicine treated plant (on shoot tip) showed positive results by judging on the cytological study. *In vitro* polyploidization that had been initiated also showed negative result. All the colchicine treated somatic embryos of *P. colubrinum* encounter blackening after the treatment. However, effort of inducing tetraploid *P. colubrinum* should be continued in future by using other antimitotic agent like oryzalin and trifluralin.

Key words: *P. nigrum* L., *P. colubrinum* Link., interspecific hybridization, artificial pollination
KAJIAN KACUKAN INTERSPESIFIK ANTARA *Piper nigrum* DAN *Piper colubrinum*

ABSTRAK

Kacukan interspesifik antara *P. nigrum* L. dan *P. colubrinum* Link. telah dijalankan dengan hasrat untuk mencapai hibrid yang tahan terhadap penyakit akar buruk (*Phytophthora* foot rot). *P. nigrum* merupakan tanaman rempah yang penting yang mempunyai kromosom tetraploid (2n=52) manakala *P. colubrinum* merupakan spesies *Piper* yang tahan penyakit *Phytophthora* yang mempunyai kromosom diploid (2n=26) dalam alam semulajadi. Objektif utama bagi kajian ini ialah untuk membuktikan keserasian dalam kacukan antara dua spesies ini. Pelbagai eksperimen telah dijalankan, merangkumi kajian biologi pembiakan bagi kedua-dua pokok induk, kajian kemungkinan berlakunya apomixes dalam *P. nigrum*, kajian pendebungaan secara artifisial dan semulajadi serta merangsangkan pembentukan *P. colubrinum* yang berkromosom tetraploid.

Kacukan interspesifik antara *P. nigrum* L. dan *P. colubrinum* Link. telah dibuktikan tidak serasi berdasarkan keputusan negatif yang telah diperolehi dalam kajian morfologi dan sitologi pada hibrid yang berpotensi dalam eksperimen pertama kacukan interspesifik dan kejayaan sifar dalam pembentukan buah lada pada eksperimen kedua pendebungaan artifisial (kaedah pendebungaan artifisial telah diubah dan diperbaiki). Eksperimen juga menunjukkan tiada percambahan debunga *P. colubrinum* pada permukaan-permukaan stigmata bagi *P. nigrum* dalam ujikaji percambahan secara in vivo. Sebelum menjalankan eksperimen kacukan interspesifik, kajian memberi tumpuan dalam penemuan kaedah pendebungaan artifisial yang berkesan antara dua spesies *Piper*. Kajian biologi pembiakan bagi kedua-dua pokok induk iaitu *P. nigrum* (jantan) dan *P. colubrinum* (betina) menunjukkan pendebungaan artifisial lebih sesuai dijalankan kira-kira bermula dari 11 pagi hingga 5 petang Dalam kajian kebolehhidupan debunga *P. colubrinum*, pembukaan...

Kata kunci: P. nigrum L., P. colubrinum Link., kacukan interspesifik, pendebungaan artifisial
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LIST OF ABBREVIATIONS

° Degree
°C Degree centigrade
% Percent
µEm⁻²s⁻¹ Micro Einstein’s per Meter squared per Second
µg Microgram
µL Microliter
µM Micromolar
µm Micrometer
a.m Approaching midday
ANOVA Analysis of variance
A.D. Anno Domini
BA N6-Benzyladenine
BAP 6-Benzylamino purine
cv. Cultivated variety
DMRT Duncan’s Multiple Range Test
DMSO Dimethyl Sulfoxide
DNA Deoxyribonucleic acid
DUS Distinctness, Uniformity and Stability
e.g For example
F₁ First generation
F₂ Second generation
F₁BC₁ First generation backcross
Fig. Figure
g Gramme
GA₃ Gibberellic acid 3
h hour
HCl Hydrochloric acid
H₂O₂ Hydrogen peroxide
HgCl₂ Mercuric chloride
IAA Indole acetic acid
IBA Indole butyric acid
ISSR Inter Simple Sequence Repeat
i.e. That is
L Litre
mg Milligram
MgCl₂ Magnesium chloride
min Minute
mL Millilitre
mm Millimetre
mM Millimolar
n.d No date
DCIB Sodium 2, 3 dichloroisobutyrate
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<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
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<tr>
<td>NAA</td>
<td>Naphthalene acetic acid</td>
</tr>
<tr>
<td>OPE 07</td>
<td>Primer kit (Sequence: AGATGCAGCC)</td>
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<td>PGR</td>
<td>Plant growth regulator</td>
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<td>pH</td>
<td>Potentiometric hydrogen ion concentration</td>
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<td>p.m</td>
<td>After the middle day</td>
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<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
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<td>Ringgit Malaysia</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<td>rpm</td>
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<td>SE</td>
<td>Standard error</td>
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<td>spp.</td>
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<td>USA</td>
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CHAPTER 1
INTRODUCTION

1.1 Introduction

Black pepper known as the 'King of Spices' is the most important and widely used spice in the world, occupying a position that is supreme and unique. The black pepper plant, *Piper nigrum* L. is a species belong to Piperaceae. It is originated from the tropical evergreen forests of the Western Ghats in India and was distributed away from the place of origin possibly by the first century A.D. or even before (Ravindran et al., 2000a).

Black pepper which was introduced into Sarawak, a state in Malaysia since 1856 (Sim, 1993) had become a major agricultural commodity of Sarawak and still plays an important role as a cash crop for smallholders until now. Currently Malaysia is one of the six largest producers of black pepper in the world. Malaysia exported 13,390 tonnes of black pepper worth RM172.1 million in year 2009 (Anon, 2009). Of these, over 90% comes from the state of Sarawak.

According to Anon (2009), black pepper was short supply by approximately 26,100 tonnes every year. Malaysia used to be the top three or four world producer of black pepper in the last century. However, black pepper production in Malaysia had not increased in the recent years. Planting of oil palms and rubber on land previously planted with black pepper is commonly seen nowadays. Constant decrease in the number of pepper growers from year to year is a phenomenon that
causes concern as this would seriously hamper black pepper industry in Malaysia in the long run. High cost of production and occurrence of pests and diseases are the main factors that discourage farmer to plant black pepper.

Among all diseases of black pepper, *Phytophthora* foot rot disease has been recognized as one of the most devastating. This disease affects all parts of black pepper plant in every stage. The fatal infection occurred when the pathogen infects the ‘collar’ or ‘foot’ (lower part of stem just below the soil) which eventually causes death of the plant. The causal agent was identified as *Phytophthora capsici* Leonian (Holliday & Mowat, 1963; Tsao & Alizadeh, 1988). In Malaysia, *Phytophthora* disease causing estimated crop losses of 5-10% annually and up to 95% for individual farmers (Sim, 1985).

Until now, there is no cultivated black pepper variety that is resistant to *P. capsici* in all black pepper producing countries (Kueh, 1978). *Piper* species of South East Asia origin tested are all susceptible to this disease (Turner, 1971 and 1973; Kueh, 1978). All cultivated black pepper varieties in Sarawak are susceptible to this disease also (Kueh, 1978; Kueh & Khew, 1980; Sim, 1983; Paulus & Sim, 1987).

However, *Piper colubrinum* Link., a species in the same genus as the cultivated black pepper originated from Puerto Rico is a shrub which shows high degree of resistance to many serious diseases of cultivated black pepper particularly the *Phytophthora* foot-rot disease (Purseglove et al., 1981). The plant had not been affected by *P. capsici* (Kuch, 1978). According to Dicto & Manjula (2007), salicylic acid (SA), the phenyl propanoid derivative of *P. colubrinum* plays a
key role in disease resistance. In Sarawak, Kueh (1978) had successfully proved the resistance of *P. colubrinum* against *Phytophthora* foot rot disease through leaf screening method. Thus, this species has good potential as a donor plant in hybridization with the cultivated species, *P. nigrum* for development of *P. capsici* resistance in cultivated black pepper.

1.2 Problem statements

The first success in interspecific cross between *P. nigrum* and *P. colubrinum* was reported by Vanaja *et al.* (2008). They reported that a partially fertile interspecific hybrid having partial resistance to *Phytophthora* foot rot was successfully developed. This hybrid was considered as a successful breakthrough for introgression of resistance to the cultivated species, *P. nigrum* from the wild species, *P. colubrinum*. However, in Sarawak, Malaysia, interspecific hybridization between *P. nigrum* and *P. colubrinum* had not been successful so far. The work initiated in 1970 (Sim, 2007) was continued only until the mid 1990s. According to Sim (2007 and personal comm.) from the programme, both erect and climbing progenies were produced. These progenies were of small stature. They were very stunted with very short internodes and all did not survive to maturity except one climbing progeny with very short fruit spikes (Sim, 2007). Leaf screening result showed this progeny had the same degree of tolerance as cv. Kalluvally to *P. capsici*. No cytological or molecular tests were conducted to verify the legitimacy of this interspecific hybrid and no further work was done on this progeny (Sim, 2007). Whilst Chen (2007) also reported success of berry set from interspecific hybridization between *P. nigrum* and *P. colubrinum*. The number of fruit setting in interspecific cross was about ¼ of that of selfing (the control for the experiment). However, the hybrid seeds were not sowed for further observation on the
morphology and for cytological analysis to ascertain the legitimacy due to uncertainty of the reliability of the artificial pollination method used. He further enlightened that even though many specialized methods or techniques had been applied, together with the advantage of protogynous nature and acropetal pattern in development of flowers in an inflorescence of black pepper, the possibility of obtaining the self pollinated fruits is still high due to very strong self pollination mechanism in pepper. Moreover, *P. nigrum* is a tetraploid with 2n=52 while *P. colubrinum* is a diploid with 2n=26 (Mathew, 1958), the possibility of interspecific cross between *P. nigrum* and *P. colubrinum* in encountering cross incompatibility is very high.

In short, there is a doubt in cross compatibility between *P. nigrum* and *P. colubrinum*. A study is needed to enlighten the practicability of carrying out interspecific hybridization between two species in future.

1.3 Objectives

As there is a doubt in cross compatibility between *P. nigrum* (tetraploid in chromosome level) and *P. colubrinum* (diploid in chromosome level), this study is thus conducted with the main objective:

- To ascertain the cross compatibility between two species. Experiment on artificial hybridization to find out the possibility of hybridization between *P. nigrum* (2n=52) and *P. colubrinum* (2n=26) was carried out. Eventually, characterization of the F1 progenies based on the morphological characteristics and cytological information were scored.
In order to achieve reliable outcome from interspecific hybridization, the following various experiments were conducted also as secondary objectives of this project:

- Study on reproductive biology of *P. nigrum* and *P. colubrinum* for the purpose of developing reliable method of artificial pollination.

- Experiment on natural and artificial pollination to verify the possible occurrence of apomixes in *P. nigrum*.

- Polyploidization of *P. colubrinum* via colchicine treatment was carried out to induce chromosome doubling to solve the problem of cross incompatibility between *P. nigrum* and *P. colubrinum*.