Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

THE ROLE OF GRAFTING IN THE RESISTANCE OF FOOT ROT DISEASE IN BLACK PEPPER (*PIPER NIGRUM*)

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ABSTRACT

Black pepper (*Piper nigrum)* is a vital spice with historical and economic significance for Indonesia. However, the cultivation of black pepper faces challenges, notably foot rot disease caused by *Phytophthora capsici.* One of the strategies to enhance black pepper resistance against foot rot disease is by grafting. By using *Piper columbrinum* as disease-resistant rootstock, it will influence the plant's defense mechanisms. The paper delves into the mechanisms of disease resistance conferred by grafting, the selection of appropriate scion and rootstock combinations, and the implications of this technique for sustainable black pepper cultivation. In this study, we evaluate the resistance of grafted black pepper against foot rot disease. It was observed that the grafted black pepper has high resistance compared to non-grafted pepper. A PCR test also showed amplification of Resistant Gene Analogs (RGAs) in grafted pepper plant infected by *Ph. capsici* while the non-grafted plant showed no amplification.

Keywords: Grafted black pepper, *Phytophthora capsici*, *Piper columbrinum*, resistant gene analog.

INTRODUCTION

Black pepper cultivation has a rich history, contributing both to culinary delights and medicinal properties. According to Directorate of distribution Statistics (2023), total export volume of blackpepper from Indonesia to the U.S were increased from 2.187 tons in 2021 to 3.368 tons in 2022. The increasing demand for this spice is due to growing consumer healthcare spending as well as a thriving pharmaceutical sectors. The plants has been proven to contain antioxidant, antifungal, antimicrobial, as well s antiepileptic (Wulandari, 2021).

However, the cultivation of black pepper faces a challange in the form of foot rot disease, caused by *Phytophthora capsici* (Ginting & Maryono, 2013). *Ph. capsici* is a soil-borne disease that can infect roots, stems, leaves, fruits of black pepper plant and also other crop plants such as tomato (Jupe *et al..,* 2013), eggplant (Naegele *et al..,* 2014), cucumber (Ando & Grumet, 2006), watermelon (Kousik *et al..,* 2018), squash, as well as other 45 species of cultivated plants and weed (Hausbeck and Lamour, 2007).

As a strategic response to fortify black pepper against this pervasive threat, grafting emerges as a promising technique. This study explores the utilization of resistant piper species such as *Piper columbrinum* (Hidayat *et al..,* 2021; Nguyen *et al..,* 2020), *Pp. betle* (Nguyen *et al..,* 2020), *Pp. hispidum*, *Pp. aduncum* and *Pp. tuberculatum (Crasque et al.., 2021)* as a disease-resistant rootstock, influencing the intricate defense mechanisms of black pepper.

Submitted: December 14, 2023
Revised: April 05, 2024
Accepted for Publication: May 15, 2024
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Within the context of this investigation, we delve into the empirical evaluation of grafted black pepper's resistance to foot rot disease. Our observations reveal a notable enhancement in resistance compared to non-grafted pepper, supported by PCR tests demonstrating the amplification of Resistant Gene Analogs (RGAs) in grafted pepper plants infected by *Ph. capsici*, while non-grafted plants exhibited no such amplification. This exploration underscores the potential of grafting as a transformative approach in safeguarding Indonesia's black pepper industry against the pervasive threat of foot rot disease.

MATERIALS AND METHODS

Plant Materials: Two black pepper varieties, *Piper nigrum* cv. 'Natar 1' and *Piper nigrum* cv. 'Natar 2' were susceptible to foot rot caused by *Phytophthora capsici* was used as a scieon. A *Piper columbrinum* known for its resistance to *Ph. capsici* was used as rootstock. The combination of experimental plants used in this study were shown in figure 1.

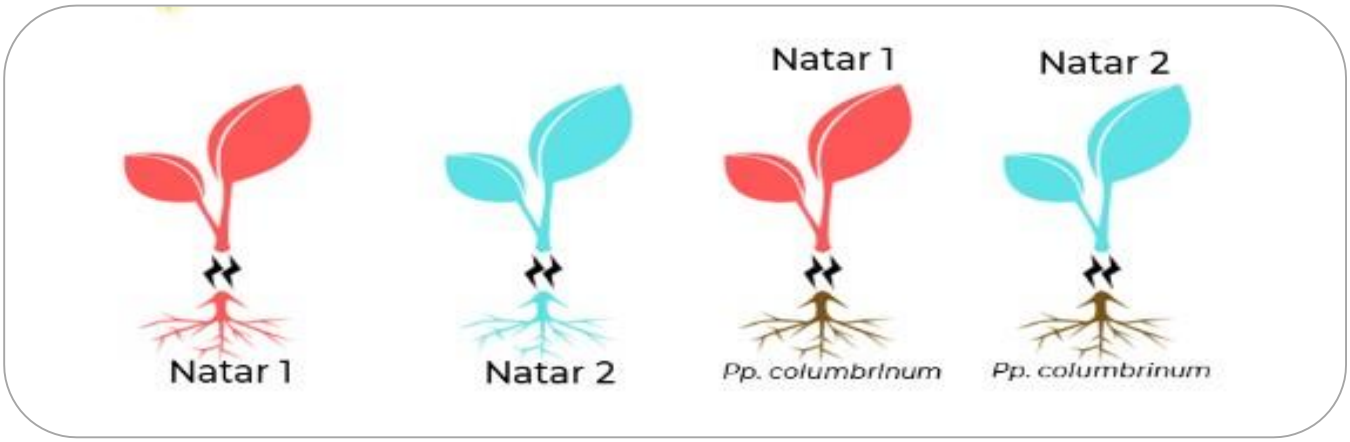

Figure 1. Black pepper varieties used in this study. (A) *Piper nigrum* cv. 'Natar 1'; (B) *Pp. nigrum* cv. 'Natar 2'; (C) *Pp. nigrum* cv. 'Natar 1' as scion and *Pp. columbrinum* as rootstock; (D)) *Pp. nigrum* cv. 'Natar 2' as scion and *Pp. columbrinum* as rootstock

***Phytophthora capsici* Inoculation:** The *Ph. capsici* were collection of Biotechnology Laboratory, Faculty of Agriculture, University of Lampung. Sixty-two days old seedlings were inoculated by spraying the root area with 10^5 zoospores/mL. Each experimental unit consisted of three plants. The leaf samples were collected at 24 and 72 hrs after inoculation. A descriptive type of assessment key was used for disease severity determination at 3 days after inoculation. The score scale was represented by 0=0%, 1=1-25%, 2=26-50%, 4=76-100% (Abraham *et al.,* 1996).

Resistant Gene Analog Amplification: The plant DNA was extracted from the leaves at 24 and 72 hours after inoculation using CTAB method explained by Ashokumar *et al.* (2020). For each sample, 1 g of leaf sample was weighed and cut it into small pieces, and 3 ml of preheated (5 min preheated at 60°C) extraction buffer (20mM EDTA pH 8, 100mM Tris HCL pH 8, 2% w/v CTAB and1.4M NaCl) was added in the pre-chilled pestle and mortar for fine grinding and homogenized after the addition of 25 mg of PVP, 25 mg of sodium metabisulphate, and 20 µl of 2-mercaptoethanol. One ml of homogenate was transferred into the 2 ml centrifuge tube, and after gentle shaking, incubated the tube for 45 min at 60°C with intermittent shaking every 10 minutes and cooled to room temperature. 1 ml solution of chloroform: Isoamyl alcohol (24:1) was added to it and mixed gently by inverting the tube 25 times to form an emulsion (Ashokkumar *et al..,* 2020). Centrifugation was performed at 10.000 rpm for 15 minutes. The supernatant then transferred to a new eppendorf tube. A 200 µL of 5M NaCl was added and mixed well. After that, 800 µL of ice- cold isopropanol was added and kept overnight in -20 °C. The DNA pellet was obtained by centrifugation at 5000 rpm for 3 minutes followed by 10.000 rpm for 3 minutes. The supernatant then discarded, and the pellet was washed with 200 µL pf 70% ethanol. The pellet then centrifuged once again at 10.000 rpm for 3 minutes. The ethanol poured off and the DNA were dried by air drying at room temperature for 30 minutes. The DNA pellet then re-suspended with 200 µL of 1X TE buffer and stored at -80 °C.

The RGA was amplified by PCR using primers described by Suraby *et al..* (2020) (Table 1). The 25 µL reaction mixtures contained 1 µL DNA, 2 uL of each forward and

reverse primer, 0.4 mg/mL Bovin Serum Albumin (BSA), 0.4 mM dNTPs (0.125 U Takara Taq DNA Polymerse (Takara Bio, Kusatsu, Japan) and PCR buffer. The PCR reaction was carried out in a BioRad Mycycler. The amplification condition was 94 ^{o}C for 10 min, 35 cycles of 94 ^{0}C for 10 s, 60 ^{0}C for 30 s and 72 ^{0}C for 30 s. The PCR result was then visualized by using electrophoresis in 1.5% (w/v) agarose gel (Suraby *et al.,* 2020).

Table 1. The *Piper nigrum* resisant gene analog primers used in this study

Primer	Sequence	Amplicon length (bp)
PnRGA1	F: GCGTTTCCAAGGACTTCACTA	160
	R: GCTTCCCATACATCATCTAGCA	
PnRGA3	F: GATGTACACCCTCACAGTCGTG	199
	R: CAGGCTGCATCTGTGAGCATAC	
PnRGA8	F: CCTCTAACTTCAAAGGCCTCTCTAC	174
	R: GTAAGGCTAGTTTCCTCTGGCTTC	
PnRGa24	F: GATGATTTGTGGGATCGTGATG	170
	R: CATGAATGCCAAGAGCTAAGAG	

RESULT AND DISCUSSION

Growth and mortality in response to *Phytophthora capsici* of black pepper grafts: Foot rot disease symptoms were observed in both non-grafted black pepper. The grafted black pepper with *Pp. nigrum* as rootstock showed no symptom of foot rot disease. The symptoms observed were leaves yellowing, wilting pepper vine and collar rot (Figure 2). The data were shown at table 2.

Table 2. Disease severity based on score scale.

Plant	Disease Severity score days after inoculation							
	1	2	3	4	5	6	7	8
Natar1	0	1	2	2	2	3	3	4
Natar2	0	1	2	2	3	3	4	4
Natar1-*Pp. columbrinum*	0	0	0	0	0	0	0	0
Natar2-*Pp. columbrinum*	0	0	0	0	0	0	0	0

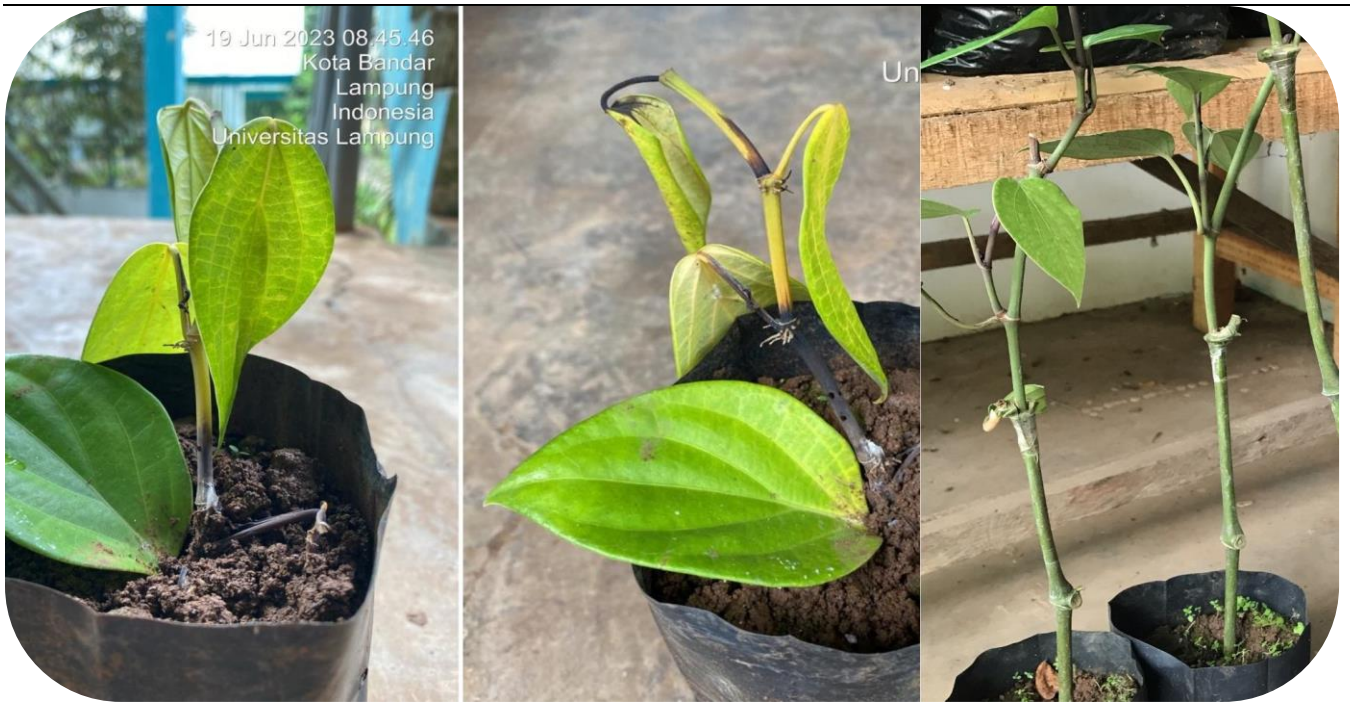

Figure 2. The black pepper plant condition at 5 days after inoculation. (A) Natar1 and (B) Natar2 showing moderate symptoms of *Ph. capsici* infection. While the grafted black pepper with *Piper columbrinum* as rootstock showing no symptoms of infection.

One of the most significant black pepper diseases in the world is foot rot disease caused by *Ph. capsici*. It is an oomycete soil-borne plant pathogen that affects a wide range of vegetables, causing foliar, fruit, and root diseases. When this virus infects plants, they die in two to three weeks in the rain, and neighboring plants become affected in one to two months (Wahyuno *et al..,* 2010).

Phloem and xylem are destroyed when a collar rotted, which stops water and nutrients from moving from the roots to the plant's aerial sections (Brown & Brasier, 2007). As a result, the plant withered halfway and showed signs of abrupt leaf wilting and falling. Crucially, farmers and technicians frequently miss the early symptoms of the disease as they are so difficult to recognize. When the upper portion of the pepper vine exhibits symptoms like leaf yellowing, withering, and dropping, they have identified the illness (Ton *et al..,* 2005). When these symptoms appear, the infection has progressed to a severe level, with the majority of the root rotting and a brownish-black lesion visible on the underground stem.

This study shows that grafting black pepper with *Pp. columbrinum* that resistant to *Ph. capsici* could suppress the pathogen infection. This result was correlated with recent study conducted by Ferrari *et al.* (2023) which grafted *Pp. nigrum* 'Bragantina' onto four wild piper species to suppress *Fusarium* infection.

Resistant gene analog amplification: The resistant gene analog was amplified using PnRGA primers described by Suraby *et al.* (2020) (Table 1). Plant disease resistance gene analogue (RGA) markers are used to map resistance genes and were created based on the conserved sequence of known RGAs (Ren *et al..,* 2013). The PnRGA were involved in pathogen recognition either by direct interaction with the pathogen effector or with pathogen modified host protein during the invsion and subsequent activation and signaling pathways leading to defense against pathogen (Gururani *et al..,* 2012). In the previous study, the PnRGA 24 activity were significantly high in *Pp. columbrinum.* This study showed that the PnRGA 24 gene were amplified in grafted blackpeper plant with *Pp. Columbrinum* as rootstock and *Pp. nigrum* cv. Natar1 and Natar2 as scion at 24 an 72 hours after innoculation. The PnRGA1 gene also amplified in *Pp. nigrum* cv. Natar1 at 72 hours after innoculation (Figure 3, Table 3).

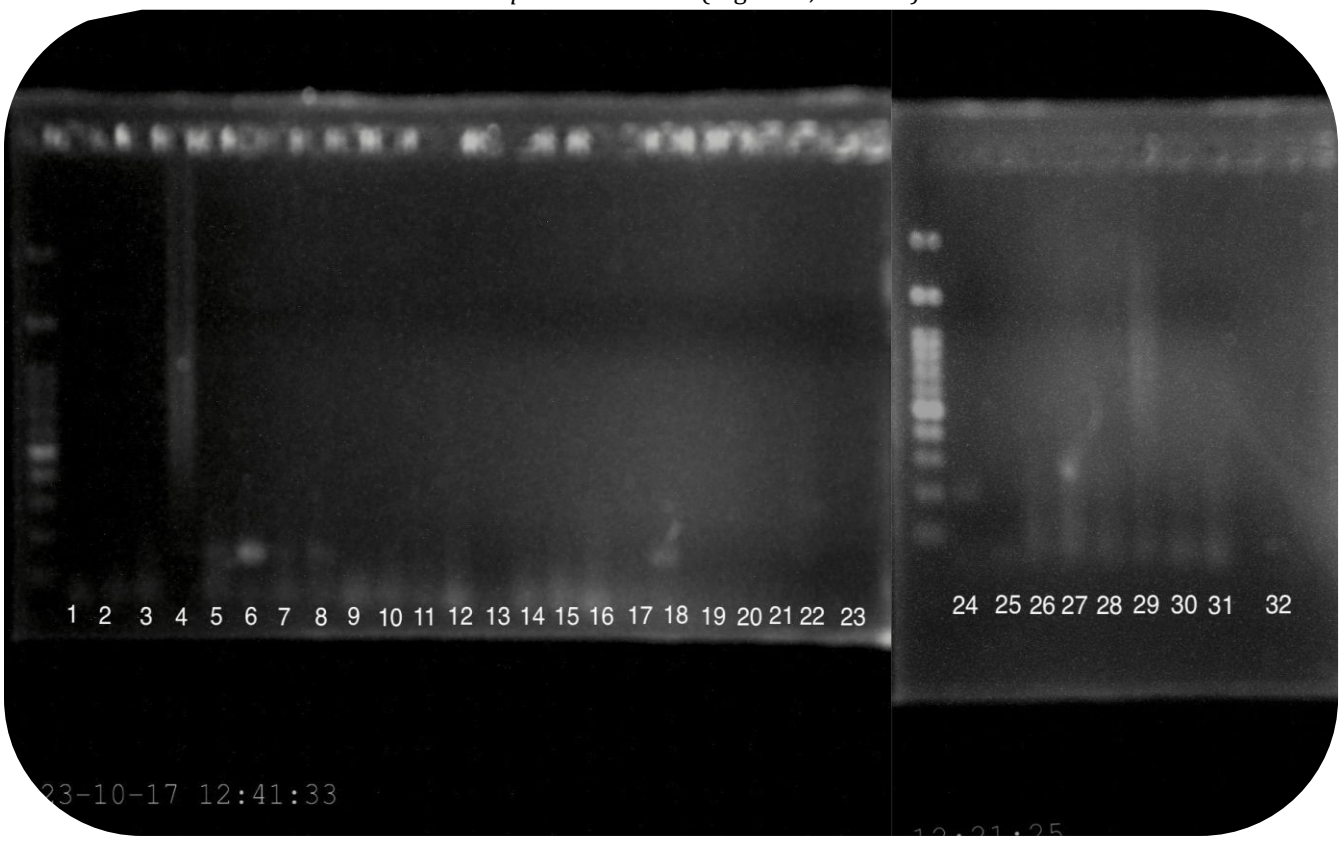

Figure 3. Agarose gel electrophoresis of PCR products for *Piper nigrum* resistant gene analog (PnRGA) primers

Table 3. PCR amplification result of *Piper nigrum* Resistant Gene Analog primer sets

Plants	Amplification							
	PnRGA1		PnRGA3		PnRGA8		PnRGA24	
	24 HAI	72HAI	24HAI	72HAI	24HAI	72HAI	24HAI	72HAI
Natar1	-	-	-	-	-	-	-	-
Natar2	-	-	-	-	-	-	-	-
Natar1-*Pp columbrinum*	-	+	-	-	-	-	+	+
Natar2-*Pp columbrinum*	-	-	-	-	-	-	+	+

HAI: Hours after inoculation

Studying a plant's resistance responses during an attempted infection can be done by contrasting the responses in susceptible and tolerant/resistant genotypes under pathogen stress and without. Certain genotypes of *Pp. nigrum* are sensitive to *Ph. capsici*; a lesion with progressive borders emerges in the leaf within 24 hours after innoculation, and the leaf falls after 72 hours after innoculation. Therefore, following pathogen exposure, a time range of 0-72 hai was selected for RGA expression investigations in *Piper* spp.

Black pepper is mostly infected with *phytopthora* through soil and aerial infections. According to Zakaria *et al.* (2022), aerial infections affect the foliage, shoots, spikes, and branches, resulting in blight, spike shedding, and defoliation, all of which cause the plant to die. Black pepper wilt is caused by an aerial infection of the plant that enters through runner shoots or roots near soil level. The aerial infection of fields caused by rain splashes and wind-blown water droplets spreads the disease quickly, while root infections leading to collar rot require a long incubation period of approximately two to three rainy seasons (Rini & Remya, 2020)

CONCLUSION

The result of the present study showed that grafting black pepper with *Piper columbrinum* as rootstock were able to develop resistance varieties towards *Phytophthora capsici*. The amplification of *Piper nigrum* resistant gene analog (PnRGA) showed activity of PnRGA 24 and PnRGA1 in grafted black pepper.

ACKNOWLEDGEMENT

This work was supported by Rumah Program Bibit Unggul Pertanian dan Pangan, National Research and Innovation Agency No. 9/III.11/HK/2023.

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Latifa Nuraini	:	Conceived and design the analysis, wrote the paper
Kristina Dwiatmini	:	Conceived and design the analysis, wrote the paper
Intan G. Cempaka	:	Conceived and design the analysis, wrote the paper
Siti Khodijah	:	Data collection and analysis
Auliana Afandi	:	Conceived and design the analysis, data collection and analysis, wrote the paper