Jurnal Perlindungan Tanaman Indonesia, Vol. 5, No. 2, 100–107, 1999

TOTAL PROTEIN CONTENT AND PATHOGENESIS RELATED-PROTEINS IN LEAF INTERCELLULAR FLUID OF CIGAR TOBACCO CULTIVAR H-877 INFECTED WITH TOBACCO MOSAIC VIRUS (TMV)

KANDUNGAN PROTEIN TOTAL DAN PROTEIN PENYEBAB PATOGENESIS DALAM CAIRAN ANTAR SEL TEMBAKAU CERUTU KULTIVAR H-877 YANG TERINFEKSI TMV

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ABSTRACT

The reaction of hypersensitive *Nicotiana* species or cultivar (*N. glutinosa, N. tabacum* cv. Xanthi nc., *N. tabacum* cv. H-877) to three isolates of TMV from Jember showed variable symptoms. Pattern of total protein from leaf was hard to distinguish between cultivar or species. The intercellular fluids (IF) of leaf tissues which contained pathogenesis related (PR) proteins of cigar tobacco cultivar H-877 (it derives the N-gene from *N. glutinosa*) has lost several protein bands than that of *N. glutinosa*. The H-382 cultivar which systemic reaction to TMV has a band with very low molecular weight, which was not detected in other species or cultivar infected with three TMV isolates. It seems there was an effect of longterm storage of leaf before IF-extraction to the resolution of some PR-protein bands in SDS PAGE. Analysis of PR-proteins of H877 cultivar in 2D-SDS PAGE showed it consist of b-4, b-5, b-6a, b-6b, b-7a, and b-8a proteins of Parent and Asselin (1984).

Key words : TMV symptoms, *Nicotiana* sp., resistance mechanism , PR-proteins

INTISARI

Reaksi jenis atau kultivar Nicotiana *yang hipersensitif* (N. glutinosa, N. tabacum cv. Xanthi nc., N. tabacum *cv. H-877) terhadap tiga isolat TMV dari Jember menunjukkan gejala yang bervariasi. Pola protein total dari daun antar kultivar dan jenis berbeda tetapi kurang jelas. Meskipun daun tembakau cerutu kultivar H-877 mewarisi gen N dari* N. glutinosa., *pada native-PAGE diketahui ada beberapa pita protein penyebab patogenesis (PR-protein) asal cairan antarsel (IF) yang tidak selengkap milik* N. glutinosa. *Kultivar H-382 yang bereaksi sistemik terhadap TMV memiliki pita PR-protein dengan bobot molekul rendah dan pita ini tidak terdapat pada jenis atau kultivar lain yang terinfeksi isolat TMV. Ada pengaruh lama penyimpanan daun sebelum ekstraksi IF terhadap resolusi beberapa pita PR-protein pada SDS-PAGE. Analisis pada 2D-SDS PAGE menunjukkan bahwa PR-protein kultivar H-877 mengandung protein b-4, b-5, b-6a, b-6b, b-7a (Parent and Asselin, 1984).*

Kata Kunci: Gejala TMV, Nicotiana *sp., mekanisme ketahanan, protein penyebab patogenesis*

INTRODUCTION

Some *Nicotiana* sp. produce necrotic local lesion if infected by pathogen, *i.e.* by virus. The production of local lesion is known as defence mechanism to this infection (Allen, 1959). This type of mechanism is a morphological phenomenon and also an immunological reaction, known as hypersensitive reaction (HR) (Müller, 1959). The HR *Nicotiana* sp., *i.e. N. glutinosa* which has the N-gene

for resistance to TMV, is also sensitive to high temperature. If the inoculated plant with TMV is maintained at temperature over 32°C, the symptoms develop to necrotic systemic infection (Linhorst, 1991; Matthews, 1991). The N-gene is known to induce some proteins that play the important role in defense mechanism to TMV infection. This protein is detected as pathogenesis related (PR-) protein and presents in the intercellular fluid of leaves. The common strain of TMV (U_1) is able to infect many dicotyledonae plants and spread all over the world (Sielgel, 1959). In Indonesia there are at least three symptom types of TMV, however, the identification of this virus into strains has never been done (Semangun, 1991).

PR-protein generally occurs in stressed plant, *i.e.* by physical or chemical treatment or by pathogen infection. The local lesion produced by virus contains either basic or acidic PR-protein which can be extracted from either intra or intercellular space. Salicylic acid (SA) is known as one of the agent that may induce a systemic acquired resistance (SAR). The SAR-gene may include the PR-1 gene (encoding a protein of unknown function), the PR-2 gene (encoding of β -1,3 gluconase), PR-3 gene (encoding chitinase) and PR-5 gene (encoding thaumatin-like protein) (Wahyuni, 1999; Maleck & Dietrich, 1999). These enzymes activity only work in response to the pathogen infection or wounding, it may hydrolyse glucan or/and chitin present in the outer cell wall (Linhorst, 1991; White & Antoniw, 1991). The kind of PR-proteins in tobacco Samsun NN and Xanthi nc. have been completely determined, however, not all of the function of PR-protein in the plant defense mechanism has been known yet. This paper presented the reaction of *Nicotiana* cultivar or species to three isolates of TMV from Jember, to compare the pattern of total protein of leaf of these infected plants, and to identify the kind of PR proteins in commercial cigar tobacco of *N. tabacum* cv. H-877.

MATERIALS AND METHODS

Plants and virus isolates. Plants used in this experiment were *N. glutinosa, N. tabacum* cv. Xanthi nc., *N. tabacum* H-877 which derived the N-gene from *N. glutinosa* (Hartana, 1987), and *N. tabacum* cv. H-382 which produces systemic infection to TMV. Both H-877 and H-382 are Besuki cigar tobacco cultivars.

Virus isolates were collected from tobacco plants grown in PT Perkebunan Nusantara X, Ajung, farmer's plant in Slawu and in Antirogo. Plants at 36 days after transplanting were inoculated with these isolates mechanically with inoculum of infected leaf crude extracted in phosphate buffer. Thirty and fifty four hours later the symptoms were observed. Leaf was harvested and kept immediately in –4°C for intercellular fluids (IF) extration.

Total protein extracted from infected leaves. Leaf infected tissues was ground with extraction buffer (2 mM Tris-HCl, pH 7.8, containing 0.5 M sucrose, 10 mM MgCl, 10 mM $CaCl_2$, 0.5 mM PMSF, 5 mM 2-mercaptoethanol), in the presence of liquid nitrogen. The extract was centrifuged at 10,000 × g for 10 min, then supernatant was precipitated in 40% (v/v) ammonium sulphate, and centrifuged again. Precipitate was dissolved in 100 ml Tris buffer (0.05 M Tris-HCl buffer, pH 7.5 containing 0.05 M $CaCl_2$), then it was passed through a DEAE-fibrous column which was equilibrated with 20 ml of Tris buffer to collect the total protein.

IF extraction from necrotic local lesion leaf. The IF was extracted according to Parent and Asselin (1984). Freshly collected leaves were cut surrounding necrotic tissues into 1–2 cm^2 then

infiltrated *in vacuo* for 5 minutes with a large of excess of cold extraction buffer. Pieces were gently blotted to dry and rolled up then placed into pinned-bottom of Eppendorf microfuge sitting on the top of another Eppendorf microfuge tube. After centrifugation at 4,000 × g, for 10 min at 4°C, the fluid was collected in microfuge tube and immediately frozen. Before electrophoresis, IF extract was concentrated with 20% TCA and kept in −20°C overnight then centrifuged at 10,000 × g for 10 min at 4°C. Precipitate was dissolved in sample buffer. Determination of the kind of PR-protein bands and its molecular weight referred to the molecular weight of PR-protein bands of *N. glutinosa* of Parent & Asselin (1984) and Parent *et al.* (1985).

Electrophoresis one dimension and two dimensions. Analysis of PR-protein from IF with one dimension electrophoresis was done according to Parent & Asselin (1984) with modification. The 12.5% or 14% discontinuous PAGE with SDS was used in this experiment. The IF sample was mixed with 4 × sample buffer {128mM phosphoric acid, 240 mM Tris HCl pH 6.7 containing 20% (w/v) sucrose and 0.04% (w/v) bromophenol blue}. Samples were electrophoresis at 50 V for 45 min then at 120 V until bromophenol blue reached closely to the bottom of gel.

The two dimensions electrophoresis was done according to Hogue & Asselin (1984) with gradient of 8–16% LiDS. In another experiment, gradient of 8–16% SDS was used instead of LiDS. A slice of the first dimension gel was cut and equilibrated in sample buffer containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, in test tube for 1h. Immersed test tube in boiling water for 3 mins then place this piece of gel on the top of two dimension gel. The electrophoresis was run for 22 h on 30 V at room temperature.

RESULTS AND DISCUSSIONS

Symptoms expression. As with the general test for TMV identification, the three isolates of virus (isolate 1 from Slawu, isolate 2 from Antirogo and isolate 3 from Ajung) all induced local lesion on hypersensitive plants such as *N. glutinosa*, H-877 cultivar and tobacco Xanthi. This indicated that the virus isolates were TMV. The reaction of *Nicotiana* species or cultivars to three TMV isolates were variable as shown on Table 1. The size of local lesion among cultivar or species also vary. The local lesion size of *N. glutinosa* was very tiny on 30 h.a.i., then developed to 0.5–0.7 cm on 48 h.a.i. on tobacco Xanthi, the local lession developed to 1 cm in diameter (Fig. 1D) then developed to necrosis on the whole inoculated leaf (Fig. 1 E, F). When the hypersensitive tobacco H-877 cultivar inoculated with isolat 3 (which causes a mosaic burn in the field) produced the maximum size of lesion of 0.6–0.8 cm (Fig.1A) then developed to systemic necrosis after 3 days inoculation (Fig. 1B) and caused the plant died. In 5–6 days later, the systemic necrosis on inoculated leaf of H-877 cultivar with isolates 1 and 2 developed to systemic necrosis in the subsequence leaves then to the stem and tips. It seems that isolate-3 has strong pathogenicity, as shown with the death of plants 3–4 days after inoculation [d.a.i.] (Table 1). This result was also reproducible in the second inoculation experiment. It is uncertain yet, the death mechanism of these hypersensitive plants, it could be caused by a strong pathogenicity of isolate, plant's age and high temperature in glasshouse when inoculation was done. Therefore, for extraction of IF purpose, the leaf samples were collected when leaf showed a small local lesion, on 30–54 h.a.i.

Table 1. Symptoms development on species or cultivar of *Nicotiana* (36 days after transplanting) inoculated with TMV isolates.

Nicotiana species or cultivar	Symptoms induced by TMV					
	Isolate 1		Isolate 2		Isolate 3	
	30 h.a.i.	54 h.a.i.	30 h.a.i.	54 h.a.i.	30 h.a.i.	54 h.a.i.
N. tabacum cv Xanthi nc	ll	ll	ll	ll	ll, snl	snl, nst
N. tabacum cv. H877	ll	ll, nst	ll	ll, nst	ll, snl	snl, nst
N. tabacum cv. H382	x	x	x	x	x	sm
N. glutinosa	ll	snl	ll	ll	ll, snl	snl, nst

h.a.i = hours after inoculation, ll = local lesion on inoculated leaf, snl = systemic necrotic lesions, nst = necrotic systemic on stem and tip, sm = systemic mosaic, x = no symptoms yet

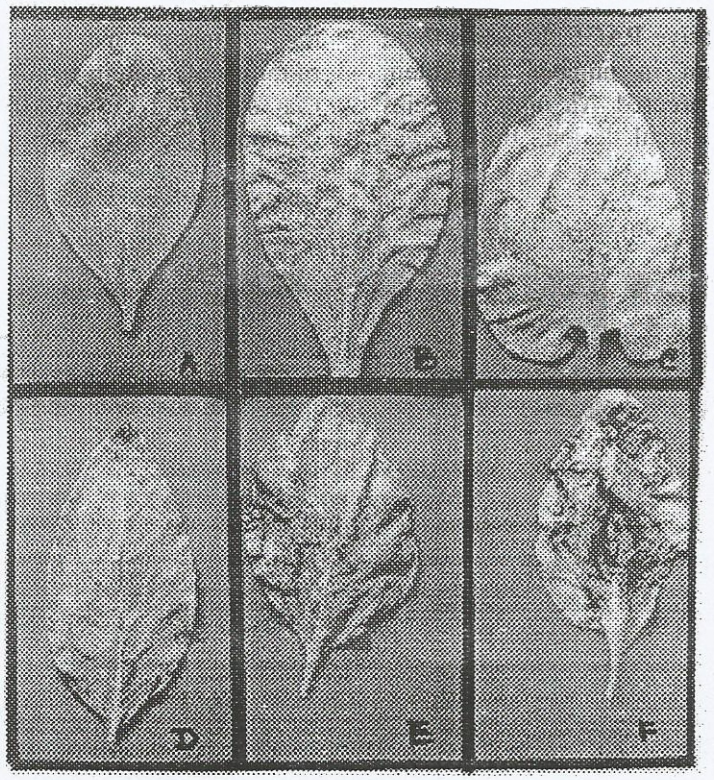

Figure 1. Variation of local lesion on hypersensitive plants A, *N. tabacum* cv. H-877 on 30 h.a.i. and B, on 54 h.a.i.; C, *N. glutinosa;* D, E, *N. tabacum* cv. Xanthi nc. on 30 h.a.i. and F, on 54 h.a.i.

IF extract. The IF extracts obtained from leaf with small local lesion (30–54 h.a.i.) showed greenish yellowing to dark brown in colour, particularly with *N. glutinosa* extract. The brown colour was due to the high level oxidation of phenolic compound in leaves. The broken tissues during the extraction procedure caused the greenish color of fluid. The volume of IF extract after infiltration with IF extraction buffer was 400–500 µl/1.5 g tissues, with protein concentration of 0.65 µg/ml. Therefore, before electrophoresis, the protein content in IF-extract was concentrated with 20% TCA or acetone (1:1, v/v).

Analysis of total protein in native-PAGE. Figure 2 showed the total protein of both healthy and infected leaf samples in native-PAGE contained fragments of the small protein subunits of enzyme Rubisco with the molecular weight of 14 kD (band no. 1) and the large protein subunits with molecular weight of 58 kD (band no. 9). These protein subunits came out from the broken cell wall when grinding sample. There was a little different in number of bands of total protein content in both infected and uninoculated leaf of hypersensitive plants, but it was hard to distinguish in native-PAGE. It seems there was no different in number of protein bands among infected *Nicotiana* species which produce local lesion, but it was different in the H-382 cultivar which produces systemic infection (Fig. 2D).

Analysis of PR-protein from IF extract on SDS-PAGE. IF-extract samples obtained from both leaves showing necrotic local lesion and systemic infection were electrophoresis in 12 % SDS-PAGE. The kind of PR-protein designated as b-protein was determined according to Parent & Asselin (1984). Fig. 3 showed that band no. 1 corresponded to b1, band no. 2 to b2, band no. 3 to b3 of Parent & Asselin (1984), etc. On H-877 cultivar (Fig. 3 C,F), the number and the electrophoretic mobility of protein bands was different to that of H-382 cultivar (Fig. 3 E) and *N. glutinosa* (Fig. 3D).

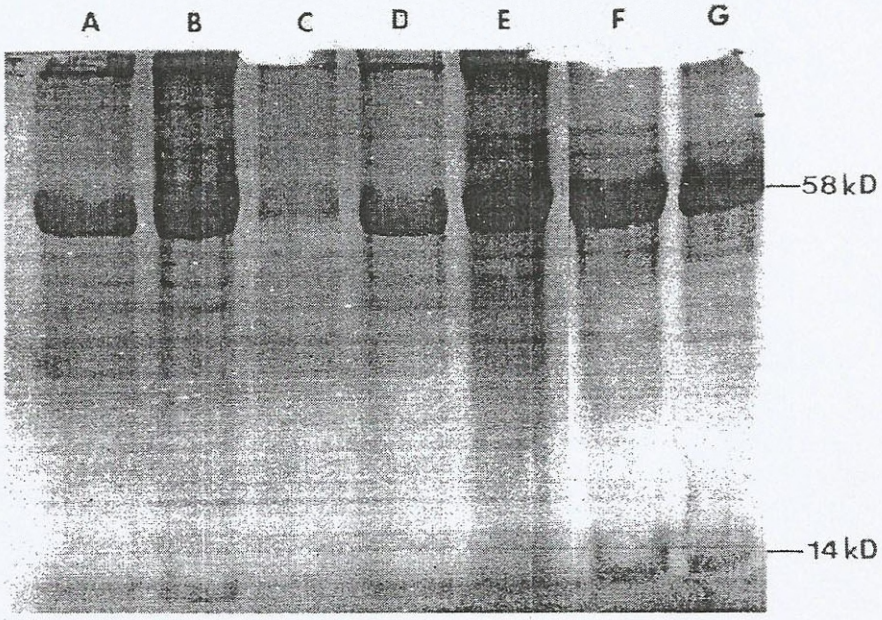

Figure 2. Analysis of total proteins of infected leaf on native-PAGE. A, uninoculated *N. glutinosa*; B, inoculated *N. glutinosa* with isolate-2 TMV (30 h.a.i.); C, inoculated *N. tabacum* cv. Xanthi nc. with isolate-1 (30 h.a.i.); D, inoculated *N. tabacum* cv H-382 with isolate-3 (30 h.a.i.); E, inoculated *N. tabacum* cv. H-877 with isolate-3 (30 h.a.i.); F, inoculated *N. tabacum* cv. Xanthi nc. with isolate-3 (54 h.a.i.); G, inoculated *N. tabacum* cv. H-877 with isolate-1 (54 h.a.i.).

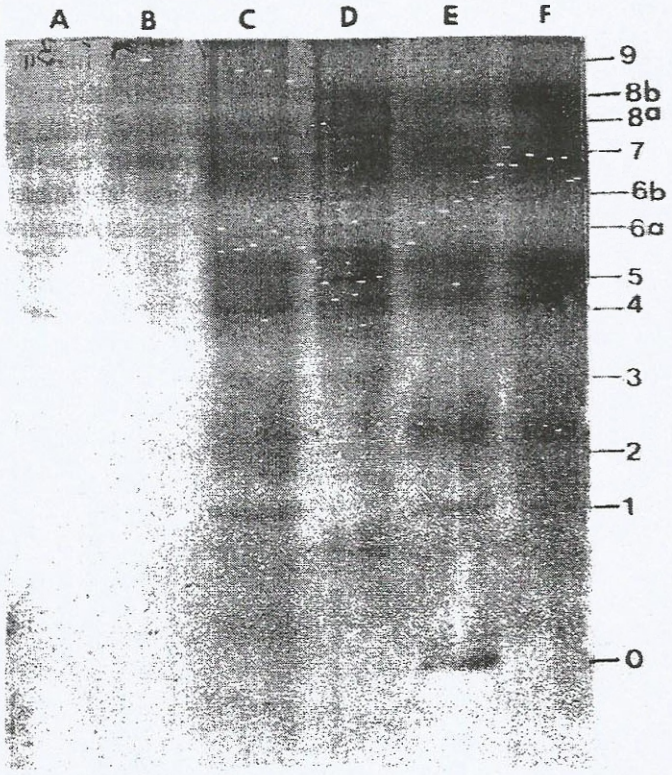

Figure 3. Analysis of PR-protein on 12% SDS-PAGE. A, uninoculated *N. glutinosa* (54 h.a.i.); B, inoculated *N. glutinosa* with isolate-2 TMV (54 h.a.i.); C, inoculated *N. tabacum* cv H-877 with isolate-3 (30 h.a.i.); D, inoculated *N glutinosa* with isolate-3 (54 h.a.i.); E, inoculated *N. tabacum* cv. H-382 with isolate-2 (54 h.a.i.); F. inoculated *N. tabacum* cv. H-877 with isolate-3 (54 h.a.i.).

Although H-877 cultivar derives the N gene from *N. glutinosa*, the band located in between the bands number 1 and 2 of *N. glutinosa* was not found in H-877 cultivar. These protein bands seemed to have an important role in the resistance mechanism to TMV infection and these were not determined in H-382 cultivar (Fig. 3 E). Pierpoint (1983 *in* Parent *et al.*, 1985) found that among species or cultivar of hypersensitive tobacco only differ in a few number of protein bands. The band number 0 in the H-382 cultivar was not found in either *N. glutinosa* (Fig. 3D) or H-877 cultivar (Fig. 3 C,F) inoculated with different TMV isolates. However, the number 0 could be a kind of plant protein that maybe induced by virus to increase polypeptide synthesis for its replication (Matthews, 1991). The longterm storage of leaf in –20°C, *i.e.* 28 days after harvest (d.a.h.) showed a difference resolution of PR-protein bands than with the leaf stored for 16 d.a.h. (data was not shown). The longer of leaf was stored, the resolution of protein bands was reduced.

Analysis of PR-protein in two dimensions electrophoresis. Result of this experiment was obtained in 7.5–15% PAGE containing a gradient of 8–16% SDS (Fig. 4). The number of b-protein of H-877 cultivar obtained from this 2D-gel electrophoresis was incomplete as the result achieved by

Parent *et al.* (1985). This could be the SDS gradient gel was not very well prepared, or because SDS used here instead of LiDS. These PR-proteins detected were b-4, b-5, b-6a, b-6b, b-7a, and b-8a, with the molecular weight of 31.2–36.7 kD. The b-4, b-5 and b-6b proteins belong to the PR-2 group of White & Antoniw (1991) that are responsible for gluconase activity. Gluconase plays the important role in the formation of proteins that resistant to protease (Parent *et al.*, 1985) and these resistant proteins may involve a part in the process of inhibition of virus replication. The inhibitor of virus replication together with PR-protein can be found in a high concentration at surrounding the local lesion (White & Antoniw, 1991; Maleck & Dietrich, 1999).

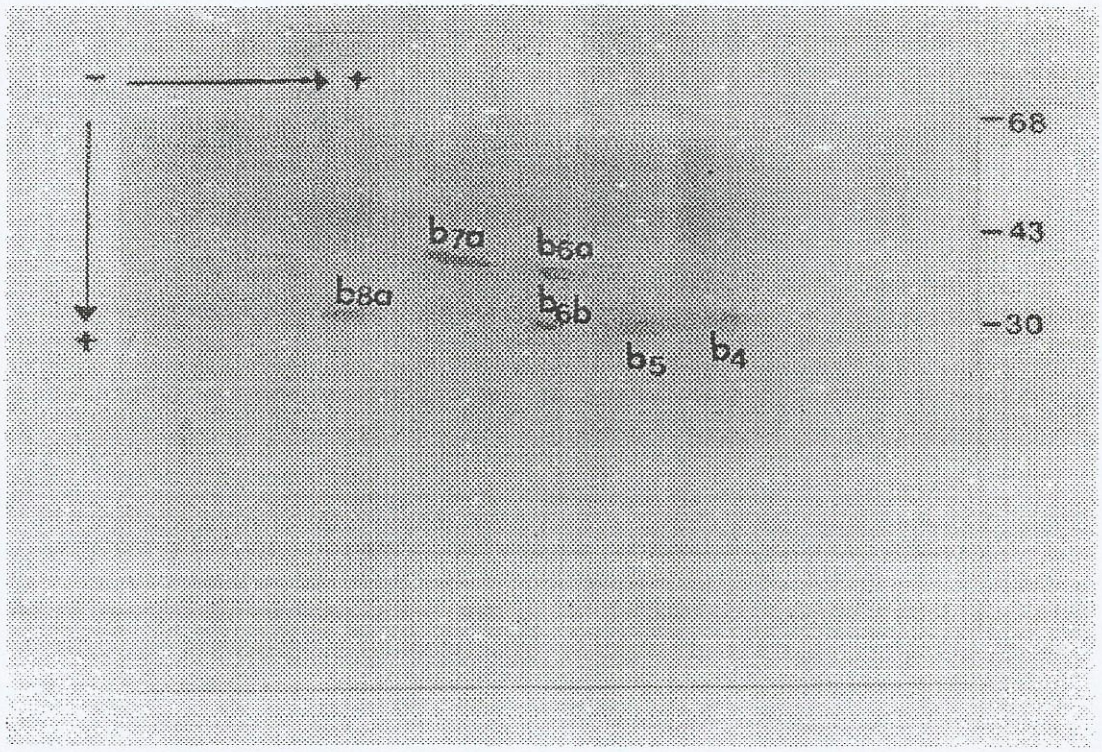

Figure 4. Incomplete pattern of b-proteins (PR-proteins) of inoculated *N. tabacum* cv. H-877 with TMV isolate-3 on 2-D SDS-PAGE. The molecular weight of b-protein referred to the results of Parent and Asselin (1984) and Parent *et al.* (1985).

ACKNOWLEDGMENT

Thank Dr. I. Hartana for providing tobacco plants, H-877 and H-382, and for the critical reading of this manuscript. This project was supported by Pengkajian dan Penelitian Ilmu Pengetahuan Dasar, the Contract Number: 20/PPIPD/DPPM/V/1998 DIKTI, Dept. P&K. RI.

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