Induction of Post-infection Ethylene and its Role in Resistance of Bean (*Phaseolus vulgaris*) against *Colletotrichum lindemuthianum*

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**Abstract**


The influence of inoculation techniques on induction of post-infection ethylene production in susceptible and resistant genotypes of bean (*Phaseolus vulgaris*) inoculated with the kappa race of *Colletotrichum lindemuthianum* was studied. Three inoculation methods (brushing, dipping and spraying) of excised leaves were compared. The brushing technique caused both the highest ethylene production at 120 h after inoculation, and better symptom development than dipping and spraying. It was, therefore, adopted to determine the post-infection ethylene production in four inoculated bean genotypes (GLP406 and Kaboon – resistant, GLP636 – moderately resistant; and MDRK – susceptible). Ethylene production increased slightly 24 h after inoculation in all four genotypes, followed by a rapid decline after 48 h. Ethylene production remained low until 120 h after which a sharp rise was observed in genotype MDRK. Increased production, though to a lesser extent, was observed in GLP636. This trend was repeatable and thus may offer an additional physiological marker to bean breeders to screen for resistance to *C. lindemuthianum*.

**Keywords:** anthracnose; bean; degree of resistance; inoculation techniques; physiological marker; race-specific resistance

Specificity of the interactions between plants and pathogens is a very comprehensive phenomenon with complicated hierarchy at different levels of biological organisation. To elucidate this phenomenon is an important task of contemporary plant pathology, including physiological and biochemical aspects (Huang 2001; Leduc et al. 2001).

Ethylene (C₂H₄) is perhaps the first known endogenous plant growth regulator. It is released by plants in very small amounts under "normal" physiological conditions and exhibits biological activity at concentrations of 1.0 ng/l (Goodman et al. 1986). Increased production of ethylene is associated with fruit ripening, flower fading, abscission, senescence of leaves and stress factors (Abeles 1972). Plant tissues produce increased amounts of ethylene, so-called stress ethylene, in response to various stress factors such as mechanical and chemical injury of plants, and to infections resulting from various host-pathogen interactions (Pegg 1976; Montalbini & Elstner 1977; Goodman et al. 1986; Boller 1991). Ethylene is generated through the “normal” biosynthetic pathway, that is via 1-aminocyclopropane-1-carboxylic acid (ACC).
Whereas studies have been carried out in many other host-pathogen systems in relation to ethylene induction, information is limited on ethylene production in beans (Phaseolus vulgaris L.) in relation to resistance or susceptibility to the bean anthracnose fungus (Colletotrichum lindemuthianum) and whether ethylene induction can be used as a marker for resistance or susceptibility in beans inoculated with the anthracnose fungus.

**MATERIAL AND METHODS**

**Plant material.** The four bean (Phaseolus vulgaris L.) genotypes were used: MDRK (Michigan Dark Red Kidney), Kaboon, GLP636 and GLP406. The origin of MDRK and Kaboon is USA and Netherlands, respectively (Balardin & Kelly 1998); they were obtained from the seed collection of the Department of Phytopathology, Technical University of Munich at Freising-Weihenstephan, Germany, where the research was carried out. The genotypes GLP636 and GLP406 are landraces originating from Kenya, and were obtained from the Gene Bank of Kenya (KARI, Muguga, Kenya). Genotypes MDRK and Kaboon exhibit susceptible and resistant reactions to the kappa race of C. lindemuthianum, respectively, whereas Kenyan genotypes GLP636 and GLP406 exhibit moderate resistance and resistance (Ombiri 2000).

Seeds were incubated in moist vermiculite in Petri-dishes to ensure good germination. The germinated seeds were planted in 10 cm diameter pots containing river sand. The pots were placed in a growth chamber and the seedlings allowed to grow for 7 d at a temperature of 20°C and relative humidity of 80%.

**Fungus isolate.** The isolate of the kappa race of C. lindemuthianum was obtained from the culture stock maintained at the Department of Phytopathology, Technical University of Munich, Freising-Weihenstephan. Inoculum was produced by growing C. lindemuthianum on Glucose Peptone Agar (GPA) slants for 2 weeks in darkness at 20–21°C. The fungus was then further multiplied by adding sterile distilled water to the agar slants. The conidia were scraped off the surface of the medium with a sterile rod to form a suspension which was then asepti-
cally added to large culture bottles with GPA that were incubated for 2 weeks in darkness at 20–21°C. Conidial suspensions were prepared by flooding the cultures with 5 ml distilled water and the conidia were scraped off the culture surface with a glass rod. Suspensions for inoculation of germinated bean seeds were filtered through cheese cloth, and the concentration adjusted to 10^6 conidia per ml using a haemocytometer. The following formula was used to adjust the inoculum to the desired concentrations (Beshir 1991):

\[ V_0 \times C_0 = V_g \times C_g \]

where:  
- \( V_0 \) – initial volume  
- \( C_0 \) – initial concentration obtained by counting on haemocytometer  
- \( V_g \) – final volume for inoculation  
- \( C_g \) – final concentration  

\[ V_g = V_0 \times C_0 / C_g \]

**Inoculation.** Three inoculation techniques (brushing, dipping and spraying) were tested for their suitability. One week old seedlings of MDRK (susceptible to race kappa) were cut at the stem and the fresh weight of the upper part with the primary intact leaves was recorded. The lower and upper surfaces of one set of five leaves were inoculated by brushing the inoculum on with a soft paintbrush. Another set of leaves was inoculated by dipping the leaves in the conidial suspension for 10 seconds. In a third set of leaves their lower and upper sides were inoculated by spraying them with an atomiser until there was runoff. As a control, distilled water was applied. The inoculated leaves were placed in specially designed plastic chambers with the cut part in the water. The leaves were arranged so that each chamber contained a pair of inoculated or uninoculated primary leaves and each treatment was replicated five times in a completely random design. Each chamber was covered with an air-tight cover fitted with a rubber-sealed outlet through which a sample of the air in the chamber could be removed. The incubation was carried out in a growth chamber at 20°C, 16 h light and 8 h darkness.

**Measurement of ethylene.** The first and second measurements of ethylene amounts were done 18 and 24 h after inoculation, respectively. Subsequent measurements were done at regular intervals of 24 h. A sample of 1 ml gas was withdrawn with a U-40-Insulin syringe. After every withdrawal of gas samples, the covers of the incubation chambers were removed and the leaves exposed to fresh air for 2 h. The concentration of ethylene in a sample (expressed in nanomoles/gram of fresh weight (nmol/g FW)) was determined using a Varian 3300 Gas Chromatograph equipped with an alumina column and a flame ionisation detector.

**Disease assessment.** Apart from ethylene measurement, disease evaluation was carried out after symptom development. Disease severity was also recorded every time an ethylene measurement was taken. Disease evaluation was carried out according to Muhalet et al. (1981) by using the following scale:

1 = healthy, no disease symptoms on the leaves;  
2 = a few isolated small lesions on the main and occasionally secondary veins of the leaf;  
3 = many small lesions scattered on the main and secondary veins, with a collapse of the surrounding tissue;  
4 = few to many large lesions scattered over the leaf surface;  
5 = many large coalesced lesions accompanied by tissue breakdown and chlorosis.

The disease severity index (DSI) was determined according to the following formula:

\[ DSI = (1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4 + 5 \times n_5) / N \]

where:  
- 1–5 – severity scales  
- \( n_1 \ldots n_5 \) – number of leaves with the respective severity scales  
- \( N \) – total number of leaves examined per treatment

**RESULTS**

**Influence of inoculation technique on induction of post-infection ethylene**

The different inoculation techniques did not significantly affect the production of ethylene 24, 48 and 72 h after inoculation (Table 1). However, significant differences were observed 96, 120 and 144 h after inoculation, with leaves inoculated by brushing showing the highest ethylene production at 120 h. Symptom development was also better on leaves inoculated by brushing compared to dipping and spraying techniques (Figure 1). Leaves inoculated by spraying had a higher DSI than those done by dipping. The uninoculated controls had a much lower ethylene production (Table 2).
Post-infection ethylene biosynthesis in bean genotypes inoculated with \textit{C. lindemuthianum}

The measurements of ethylene production showed that there was initially an increased ethylene production 24 h after inoculation and in all four genotypes, with the resistant genotypes (GLP406 and Kaboon) showing a significantly higher ethylene induction than the two other genotypes (Figure 2). However, a rapid decline in ethylene production was observed 48 h after inoculation. Ethylene production remained low until 120 h after inoculation when a sharp increase was observed in susceptible genotype MDRK and, though to a lesser extent, also in genotype GLP636. Conversely, the resistant genotypes (GLP406 and Kaboon) as well as the uninoculated controls exhibited low ethylene production (Figure 3).

The results were repeatable and indicated a significant positive correlation ($r = 0.94$; $P = 0.01$) between disease severity index and post-infection ethylene production (Figure 4). While this was not quite evident in the first 96 h, significant differences between resistant, moderately resistant and susceptible genotypes were observed 120, 144 and, to a lesser extent, 168 h after inoculation (Figure 2).

### Table 1. The influence of inoculation method on ethylene production by leaves of susceptible bean genotype MDRK inoculated with race kappa of \textit{C. lindemuthianum}

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>Ethylene production (nmol/g FW)*</th>
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<tbody>
<tr>
<td>18</td>
<td>dipping</td>
</tr>
<tr>
<td></td>
<td>1.4a</td>
</tr>
<tr>
<td>24</td>
<td>2.6a</td>
</tr>
<tr>
<td>48</td>
<td>0.8a</td>
</tr>
<tr>
<td>72</td>
<td>0.5a</td>
</tr>
<tr>
<td>96</td>
<td>5.3a</td>
</tr>
<tr>
<td>120</td>
<td>10.5a</td>
</tr>
<tr>
<td>144</td>
<td>7.5b</td>
</tr>
<tr>
<td></td>
<td>spraying</td>
</tr>
<tr>
<td></td>
<td>1.6a</td>
</tr>
<tr>
<td></td>
<td>2.9a</td>
</tr>
<tr>
<td></td>
<td>0.6a</td>
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<td></td>
<td>0.6a</td>
</tr>
<tr>
<td></td>
<td>8.3b</td>
</tr>
<tr>
<td></td>
<td>18.1b</td>
</tr>
<tr>
<td></td>
<td>5.3a</td>
</tr>
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<td></td>
<td>7.7b</td>
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*aMean ethylene production of five independent incubations
*MMeans within a row followed by different letters (a, b, c) are significantly different ($P = 0.05$) according to Duncan’s Multiple Range Test (DMRT)
Table 2. The production of ethylene by uninoculated (water application) control leaves of susceptible bean genotype MDRK

<table>
<thead>
<tr>
<th>Hours after water application</th>
<th>Ethylene production (nmol/g FW)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>dipping</td>
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<tr>
<td>18</td>
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<td>24</td>
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<td>48</td>
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<td>72</td>
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<td>96</td>
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<td>120</td>
<td>0.7</td>
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<tr>
<td>144</td>
<td>0.6</td>
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</table>

* Mean ethylene production of five independent incubations
Analysis of variance (ANOVA) showed no significant differences of treatments at \( P = 0.05 \)

Figure 2. Post-infection ethylene production by leaves of bean genotypes GLP406 (resistant), GLP636 (moderately resistant), MDRK (susceptible) and Kaboon (resistant) inoculated with the kappa race of *C. lindemuthianum*

Figure 3. Ethylene production of uninoculated (water application) control leaves of bean genotypes GLP406 (resistant), GLP636 (moderately resistant), MDRK (susceptible) and Kaboon (resistant)
DISCUSSION

Various plant disease symptoms (stunting, abscission, yellowing, epinasty, hyper- and hypoplasia) caused by plant pathogens (viruses, bacteria and fungi) may be traced to altered hormone levels in infected plants. In some cases these symptoms could reflect an interaction between two or more growth regulators, e.g. the increased levels of ethylene generated at the site of virus infection may be due to elevated abscisic acid (ABA) concentrations. The increased production of ethylene is considered as a consequence of injury of host cells by infecting fungi and/or mechanical damage. However, although ethylene itself is able to damage or induce damage or senescence in plant cells (Goodman et al. 1986), from recent results the role of ethylene appears to be more complex. These suggested that the burst of ethylene occurring early in a hypersensitive reaction was responsible for initiating a signalling pathway leading to systemic acquired resistance (SAR) and its associated biochemical changes (Van Loon 2000).

Our results showed that there is strong influence of the inoculation technique on the level of induction of ethylene in beans inoculated with C. lindemuthianum. The dipping technique gave the lowest infection, and this was reflected by a low level in post-infection ethylene production. The low level of infection with this method is most likely a result of the high adhesive force between the spores and water, so that when the leaf is removed from the spore suspension only a low number of spores is left on the leaf surface, leading to low infection.

The spraying technique is the most common method to inoculate leaves (Gassana 1991). However, there are certain disadvantages that have been associated with this method. It often results in variable infection, perhaps because more spores tend to reach the upper surface than the underside of the leaf. Excessive spray may also cause the spore suspension to form larger droplets that run off sprayed leaves and consequently decrease the uniformity of infection.

The brushing technique using camel hair seems to wet the leaf more uniformly, resulting in a more uniform distribution of the spore suspension, and in a more uniform infection of leaves than by the other methods. This is consistent with the observation made by Tu (1985) where the infection was better in leaves inoculated by brushing than by either dipping or spraying. The technique may also cause some wounding of the leaves, thereby creating avenues for the spores to enter the host. In this study, the brushing technique was thus considered to be superior to either spraying or dipping. The differences in post-infection ethylene production had, therefore, more to do with the infection level which in turn depended on the inoculation technique used. While there was a more efficient infection, the brushing and possible mechanical damage of the leaf surface could by itself be one of the primary sources of increased

Figure 4. Correlation between disease severity and ethylene production of bean genotypes MDRK (susceptible), GLP636 (moderately resistant), GLP406 and Kaboon (resistant) inoculated with the kappa race of C. lindemuthianum
ethylen production. The reason is that ethylene is generally considered as a “stress hormone” of plants released after e.g. wounding and mechanical injury (GOODMAN et al. 1986).

The possible role of ethylene in disease resistance has long been discussed (PEGG 1976; GOODMAN et al. 1986; VAN LOON 2000). GOODMAN et al. (1986) concluded that ethylene was unlikely to be involved in host-pathogen compatibility or incompatibility, including a role as a key trigger in resistance mechanisms. However, recent results strongly suggest that ethylene contributes to SAR, possibly by facilitating the release, synthesis or transport of the mobile signal (VAN LOON 2000).

The post-infection ethylene biosynthesis determined in our experiments (Figures 2–4) demonstrate some role of ethylene in host-pathogen interaction and resistance of beans against C. lindemuthianum. The slight increase in ethylene production by inoculated bean genotypes 24 h after inoculation is most probably due to a hypersensitive reaction of host leaves to infection and/or may be the result of wounding at excision of the leaves. The early events of ethylene production do not show any definite relationships between the level of resistance and ethylene production. This is because the resistant genotypes exhibited the highest ethylene production at this particular stage whereas the moderately resistant genotype showed a lower level than the susceptible one. Yet this may be different in host genotypes with various resistance levels and genetic backgrounds. These aspects must be seriously considered in resistance screenings.

The observations made at 120, 144, and 168 h after inoculation, where resistant bean genotypes produced less post-infection ethylene whereas the susceptible produced more, are consistent with reports of high ethylene production during expression of susceptibility (GENTILE & MATT A 1975; PEGG & CRONSHAW 1976; WENDLAND & HOFFMANN 1987). In the present study there is a high positive correlation between disease severity and post-infection ethylene production (Figure 4). A similar phenomenon was recorded in our previous experiments on peroxidase activity (OMBIRI et al. 2002). Therefore, it seems that differences in ethylene production could be a potential and valuable indicator (marker) of resistance and be used as an additional tool to screen bean genotypes for resistance to the anthracnose fungus.

Future work should determine whether the stimulation of ethylene upon infection is primarily determined by the genetic constitution of the host plant or of the infecting pathogen. This research must involve more host genotypes with race-specific resistance/susceptibility and more avirulent/virulent races of C. lindemuthianum.

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References


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Soubren


Sledovali jsme vliv metod inokulace na postinfectní indukci tvorby ethylenu u náchylných a rezistentních genotypů fazolu obecného (Phaseolus vulgaris) inokulovaného rasou kappa Colletotrichum lindemuthianum. Na oddělených listech byly srovnávány tři metody inokulace (mechanické povrchové narušení, ponoření a postřik). Nejvyšší produkce ethylenu se projevila 120 h po inokulaci při použití metody povrchového narušení listů. Rovněž vývoj symptomů byl ve srovnání s ostatními metodami intenzivnější na listech inokulovaných pomocí této metody. Metoda povrchového narušení listů byla použita při studiu produkce ethylenu u čtyř genotypů fazolu (GLP406
a Kaboon – rezistentní, GLP636 – částečně rezistentní a MDRK – náchylný) inokulovaných rasou kappa C. lindenmuthianum. Měření produkce ethylenu prokázalo jeho mírné zvýšení 24 h po inokulaci všech čtyř testovaných genotypů. Prudké snížení produkce ethylenu se projevilo u všech genotypů 48 h po inokulaci. Následně zůstávala produkce ethylenu nízká, jeho nárůst byl u genotypu MDRK pozorován až po 120 h. V menším rozsahu bylo zvýšení produkce ethylenu pozorováno u genotypu GLP636. Uvedené trendy byly zjištěny opakovaně a proto se domníváme, že by tento jev mohl být využit jako jeden z fyziologických markerů při šlechtění fazolu na rezistenci ke C. lindenmuthianum.

Klíčová slova: antraknóza; stupeň rezistence; fazol; metody inokulace; fyziologický marker; rasově specifická rezistence

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