Control of *Fusarium oxysporum* infection in transgenic tobacco carrying oxalate descarboxilase gene

Danielle L. A. S. Amaral¹, Natália dos Anjos Pinto¹, Vinicius Carius de Souza¹, Francisco José Lima Aragão², Marcelo de Oliveira Santos¹*

¹Departamento de Biologia, Universidade Federal de Juiz de Fora, 36036-900, Juiz de Fora, MG, Brazil.
²Embrapa Recursos Genéticos e Biotecnologia, 70770-917, Brasília – DF, Brazil.

**ABSTRACT**

*Fusarium oxysporum* is a widespread necrotrophic plant pathogen. Its infection affects several crop plants such as potato, tomato, cotton, banana, coffee, strawberry and sugarcane. The fungus produces oxalic acid, which triggers apoptosis in plants by a reactive oxygen species-dependent mechanism. Tobacco plants were genetically modified to express a gene coding for the enzyme oxalate descarboxilase (*OxDc*), isolated from *Flammulina velutipes*. The transgenic plants showed tolerance to *Fusarium oxysporum*. Even under lower expression of the gene, plants were able to control *F. oxysporum* infection. Thus, we demonstrated that the expression of the *oxdc* gene is a good alternative for the development of *F. oxysporum* resistant crops.

**1. INTRODUCTION**

Significant yield losses in crop production are caused by a wide range of biotic stress factors. Fungal diseases are rated as one of the most important factors contributing to decrease in production of economically important crops [1]. *Fusarium oxysporum* is a necrotrophic fungus that causes vascular wilt in different plants species, including cotton, banana, coffee, strawberry and sugarcane. Fungal pathogens can reduce the fitness of their hosts or promote plant death [2]. Fungus control is difficult, because spores remain on the soil for a long time and may be transmitted by the mother plant in vegetative propagating cultures. Some strawberry cultivars are susceptible to *Fusarium oxysporum*. Traditional integrated pest management (IPM) strategies associated to breed tolerant or resistant cultivars may mitigate the problem [3]. The availability of resistance genes for plant transformation may overcome the problem. The symptoms presented during the plant infection include: 1) vascular wilt; 2) epinasty of the leaves and lightening of the ribs; 3) the reduction in growth, yellowing of the small leaves; 4) progressive wilting of leaves and stems; and 5) plant death [4]. *F. oxysporum* is considered a necrotrophic plant pathogen and secretes oxalic acid (OA) into the tissues of host plants during infection process. Oxalic acid is toxic to plant cells due to its chelation of Ca⁺⁺ in the apoplast, thus weakening the cell wall, depressing defense responses such as phenol oxidase activity and the oxidative burst, as well as due to lowering the pH of the apoplast, which increases the activity of some fungal cell-wall-degrading enzymes [5, 6, 7].


This enzyme reduces OA accumulation during appressorium formation. OA participates in the same pathway as Nep1-Like Peptides (NLP) do, inducing cell death by Reactive Oxygen Species (ROS) during *Moniliophtora perniciosa* infection in tobacco leaves [11]. Plants expressing oxalate decarboxylase enzyme (*OxDc*) isolated from *Flammulina velutipes* were able to degrade OA, being tolerant to fungal infection. The enzyme catalyzes the OA degradation into formic acid and carbon dioxide [12].

* Corresponding Author
Email: marcelo.santos @ uff.edu.br

© 2017 Danielle L. A. S. Amaral et al. This is an open access article distributed under the terms of the Creative Commons Attribution License -NonCommercial-ShareAlike Unported License (http://creativecommons.org/licenses/by-nc-sa/3.0/).
Tobacco, tomato, soybean and lettuce transformed with OxDC gene showed tolerance to phytopathogen Sclerotinia sclerotiorum [13, 14, 15] and to Moniliophthora perniciosa, which causes the witches’ broom disease in cacao [11]. The main objective of our work was to insert an OxDC gene from Flammulina velutipes into the tobacco plants in order to evaluate its effects on the tolerance to Fusarium oxysporum, a widespread plant pathogen.

2. MATERIAL AND METHODS

2.1 Tobacco transformation

Agrobacterium GV3101 carrying pCambiaOxDC was grown in Lysogeny Broth (LB) with 50 mg/L gentamicin and 100 mg/L kanamycin. When optical density (OD) reached values between 0.5 and 1.0, the culture was transferred to sterile 1.5 mL microtubes and centrifuged at 5,000 rpm for 10 minutes. The pellet was suspended in NaCl 0.85% and Acetosyringone (200 μM) up to 0.4 OD. Leaves of in vitro tobacco culture were cut in half and then cocultured with bacterial suspension for 48 hours. Explants were transferred to MS medium [16] supplemented with 1 mg/L 6-benzylaminopurine (BAP), 200 mg/L timentin and 10 mg/L of hygromycin and maintained on MS medium for shoot regeneration during 4-8 weeks. The vector pCambiaOxDC contains the OxDC gene under control of the 3SS CaMV promoter and the selectable marker gene hpt that confer hygromycin tolerance (Figure 1).

2.2 PCR screening of transformed plants

Plantlets of both T₀ and T₁ generations were screened by PCR for the presence of hptII and OxDC genes according Silva et al. (2011)[11]. Genomic DNA was isolated from young leafs [17]. Primers hyg 268 (5'-CTCGGAAGTGCTTGACATTGG-3') and hyg 672 (5'-ATGTTGCGACCTCGGTATTGG-3') were used to amplify a 404bp fragment from hptII gene and OXDC 873 (5'-TGGGCTCGACAGAGGAGAAG-3') and OXDC 371 (5'-CTCGGCAGCAGAATGAGGTC-3') were used to amplify a 502bp.

2.3 Progeny analysis

Seeds of the first generation (T₁) of self-pollinated plants were germinated on MS medium containing 10 mg/L hygromycin. Thirty plants of each line were screened by PCR to confirm the presence of the transgenes. The chi-square χ² analyses were performed to determine whether the observed segregation were consistent with the Mendelian ratio (3:1 or 15:1) with a 95% confidence level.

2.4 Transgenic lineages resistance test against oxalic acid

Both T₀ and T₁ generations were tested for tolerance to oxalic acid. The assay was performed according to [11]. Photosynthetically active leaves (triplicates) from wild type (WT) and transgenic plants were excised, and the petioles were immediately dipped in 20 mM OA (pH 4.0). Leaves were incubated in a growth room for 72 h at 25 ºC under a 16-h photoperiod. The extent of necrosis was recorded after 24 h and 48h of exposition.

2.5 Transgenic lineages resistance test against F. oxysporum

Both T₀ and T₁ generations of transgenic lines that presented tolerance to oxalic acid were tested for resistance to F. oxysporum. F. oxysporum employed in assays was kindly provided by Universidade Federal de Viçosa (Viçosa, MG, Brazil). The fungus was grown on PDA medium (20% potato, 2% dextrose, 15% agar). Inoculation was done according to Dickson & Hunter (1983) [18]. In order to produce the inoculum for the detached leaf assays, a single small piece of mycelium plug was placed at the center of a new PDA petri dish and the fresh culture was incubated at 20 ºC for 2 days in the dark. A mycelial agar plug was cut from the growing margins of F. oxysporum culture and applied to the adaxial surface of a detached leaf (Nine leaves from each transgenic and nontransgenic for T₀ and three leaves from each transgenic and nontransgenic for T₁). Symptoms were observed every 24 h. Lesion length records and images were used to measure the infected area using the Image Pro Plus® software. The area under the disease progress curve (AUDPC) was used to summarize the progress of disease severity [19].

2.6 RT-PCR expression analysis

Transgenic lines (T₀ and T₁ generations) were analyzed for the presence of OxDC gene transcripts. Total RNA was extracted from leaves using SV total RNA isolation system kit (Promega®). It was used to produce cDNA by employing the SuperScript® III First-Strand (Invitrogen®), according to the protocol suggested by the manufacturer. PCR reactions were carried out using the same protocol of transgene detection. The primers used were OXDC 873 (5'-TGGGCTCGACAGAGGAGAAG-3') and OXDC371 (5'-CGGCAGCAGAATGAGGTC-3') for oxalate decarboxylase detection and 18S rRNA as endogenous control (5'-GAGCTAATACGTTGCAACAAAC-3' and 5'-AGGGAAATGGCTCCTACTGTA-3').

Fig. 1: Expression cassette T-DNA from pCambia1390 containing Flammulina velutipes Oxalate Descarboxylase gene (FvOxDc) under control of Cauliflower mosaic virus and a sequence enhancer from Alfalfa mosaic virus (Adapted from Dias et al, 2006).
3. RESULTS AND DISCUSSION

3.1 Transgenic T₀ analysis

3.1.1 Tolerance to oxalic acid in T₀ transgenic lineages

After co-culture, 14 plants were regenerated, 11 lines (78.57%) revealed the presence of OxDc gene as observed in the PCR screening (Figure 2). Eight lines were used to test tolerance to oxalic acid. After 48h of exposure to oxalic acid, necrotic lesions and wilt started to appear in non-transformed plants and P3, P5, P7 and P8 transgenic lines while lines P1, P2, P4 and P6 did not show necrotic symptoms (Figure 3). These results are in accordance to (Silva et al 2011) [11] who observed different tolerance levels to oxalic acid, because these plants display varying levels of the enzyme activity.

Fig. 2: PCR of transgenic lineages of Nicotiana tabacum cv xanthi carrying OxDc gene. M (molecular marker 1 kb ladder); C+ (positive control – plasmid pCambiaoxdc); C - (negative control – water); P1 - P8 (transgenic lineages).

Fig. 3: Treatment of Oxalic acid solution 20 mM OA (pH 4.0) after 48 hours. C+ (positive control – WT plant); C- (negative control – acetic acid solution 20 mM pH4.0); P1-P8 (transgenic lineages).

3.1.2 Tolerance of T₀ transgenic lineages to Fusarium oxysporum

Detached leaves of transgenic plants were inoculated with a 1 cm² agar plugs from the growing margins of 2-day-old F. oxysporum cultures and lesion length was photographed. Lineages P1, P2, P4 and P6 showed less severe symptoms. The mycelium of F. oxysporum was able to initiate growth from the plug and attach to the leaf surface at inoculation sites of tolerant transgenic plants at the same time as in non-transgenic ones. However, a delay in colonization of the foliar tissue was observed after 42 and 66 hours post-inoculation, indicating variable tolerance. In non-transgenic plants, after 42 hours F. oxysporum was able to colonize plant tissues causing wilt. In the lines P1, P2, P4 and P6, the AUDPC displayed a significant delay in the symptom development compared to non-transgenic plants even after 90 hours (Figure 4a). In the lines P3, P5, P7 and P8, the AUDPC was not significantly different from the control (Figure 4b). These observations are in agreement to the expectations for transgenic lines, because the expression of the transgene in active chromatin is highly variable [14].

Fig. 4: Disease Progression of transgenic lineages leaves infected with Fusarium oxysporum. A) Time progression of area under the disease progress curve (AUDCP); B) C (control), P1, P2, P4 e P6; B: C (control), P3, P5, P7 e P8.

3.1.3 Expression analysis of OxDc in T₀ transgenic lineages

RT-PCR analysis was used to detect the expression of OxDc. It was carried out in lineages that were tolerant to F. oxysporum (P1, P2, P4 and P6) and the susceptible line P3. Non-transgenic plants were used as a control. The relative gene expression quantification was conducted. Transgenic lines showed variable of OxDc transcripts (Figure 5).

Fig. 5: RT-PCR of transgenic lineages T₀ of Nicotiana tabacum cv xanthi carrying OxDc gene. C - (negative control – water); P1 - P8 (transgenic lineages).

P3 lineage, which displayed severe Fusarium symptoms, presented the lowest OxDc expression level. This variation was directly associated to the severity of the symptoms in plants that
showed lower OxDC expression. Although the results were promising, the analysis of seed plant derived must be performed to confirm our results. Thus, the lines P1, P2, P4 and P6 were selected for progeny analysis.

3.2 Transgenic T1 progeny analysis

Transgenic lines that showed tolerance to oxalic acid and to F. oxysporum (P1, P2, P4 and P6) were acclimatized and allowed to produce seeds. The results of PCR screening, using hyg 268 and hyg 672 primers showed that all of them presented Mendelian segregation ratio of 3:1 (Table 1). The presence of OxDC was confirmed in the transgenic lines P1, P2, P4 and P6 using specific primers OXCD 873 and OXDC 371, and all plants showed the presence of both transgenes.

Table 1: Segregation analysis of transgenic lineages T1 of Nicotiana tabacum cv xanthi carrying OxDC gene.

<table>
<thead>
<tr>
<th>Lineages T0</th>
<th>Generation T1,a</th>
<th>Segregation ratio tested</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>25</td>
<td>3:1</td>
<td>1.11</td>
</tr>
<tr>
<td>P2</td>
<td>24</td>
<td>3:1</td>
<td>0.4</td>
</tr>
<tr>
<td>P4</td>
<td>25</td>
<td>3:1</td>
<td>1.11</td>
</tr>
<tr>
<td>P6</td>
<td>22</td>
<td>3:1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*a Data based on evaluation of the presence of HPTII gene by PCR. X² tabulated: 3.84

3.2.1 Resistance to oxalic acid in T1 transgenic lineages

Leaves from control (WT) and progeny of homozygous transgenic plants P1, P2, P4 and P6, were excised and the petioles were immediately dipped and in OA solution (20mM). After 24 hours, transgenic plants showed less severe symptoms than non-transgenic ones, but P2 symptoms were more severe than the other transgenic lineages. After 48 hours, a marked difference between P1, P4 and P6 and non-transgenic plants was observed, while P2 showed necrosis and wilt similar to non-transgenic (Figure 6), probably due to gene silencing.

3.2.2 Resistance test of T1 transgenic lineages to Fusarium oxysporum

Detached leaves of transgenic T1 plants were inoculated with 1cm square agar plugs from growing margins of 2-day-old F. oxysporum cultures and lesion length was photographed. All lineages tested, P1, P2, P4 and P6, showed tolerance to F. oxysporum infection. After 70 hours of inoculation, the mycelium of F. oxysporum was able to initiate growth from the plug and attach to the leaf surface of, P1, P2, P4 and P6, in a same manner as in T0, at inoculation sites. It happened in a similar fashion as in non-transgenic plants, but in the transgenic ones, the fungus was not able to colonize leave surface even after 90 hours of inoculation, neither was it able to cause necrosis and wilt. The delay and resistance observed may be associated to the action of oxalate descaboxylase enzyme in transgenic plants. During infection, the gene is developmentally expressed in the fungus Sclerotinia sclerotiorum. The enzyme Scodc2 induces appressorium development [10]. These authors suggested that the infection ability is probably allowed by signal transduction derived from OA accumulation after appressorium formation.

3.2.3 RT-PCR OxDC expression analysis in T1 transgenic lineages

Finally, RT-PCR analysis detected the expression of OxDC in T1 lineages, P1, P2, P4 and P6, demonstrating that similar level of expression was observed except for P2 lineage which showed lower level of transgene expression (Figure 7). A different result was found in T0 generation. Interestingly, transgene expression has been reduced due to the progressive methylation of the promoter, which may be altered during generations [20]. As observed for T0 plants, T1 showed similar results, confirming that lower expression levels of OxDC gene is directly related to tolerance symptoms.

Fusarium oxysporum is a widespread necrotrophic fungus that affects several crops. The fungus infection uses an oxidative burst that serves as a weapon; it triggers nutrient leakage from the host cells allowing the pathogen to survive from dead tissues causing significant crop losses [21]. In the present work, we observed that transgenic plants of tobacco showed different expression levels of OxDC gene, which might be associated to different fungus tolerance levels.

The expression of our transgene occurred under control of CaMV35S, a constitutive promoter, leading to production of transcript in both leaves and roots. Thus, AUCDP is a plausible quantitative test which may intuitively serve as a predictive test for root infection as well. Results produced in a recent work by Liu et al (2016) [22] showed that MYB expression under CaMV35 promoter is involved in the resistance against Fusarium oxysporum and Botrytis cinerea in roots and leaves. Thus, in future research, root resistance may be achieved in commercial valuable crops expressing the transgene OxDC gene. The plants expressing
FvOXDC showed resistance to *Fusarium oxysporum* while in vitro experiments showed that after some hours the tissue is infected since dead tissue is a substrate for *Fusarium oxysporum* development. Finally, the present work showed the potential of oxalate decarboxilase in the control of fungus infection, suggesting that the gene is a good alternative for the development of commercial tolerant cultivars and these plants may be introduced in organic culture.

### 4. ACKNOWLEDGEMENT

Foundation for Support of MG Research (FAPEMIG); National Council of Scientific and Technological Development (CNPq).

**Conflict of Interests:** There are no conflicts of interest.

### 5 REFERENCES


**How to cite this article:** Amaral DLAS, Pinto NA, Souza VC, Aragão FJL, Santos MO. Control of *Fusarium oxysporum* infection in transgenic tobacco carrying oxalate decarboxylase gene. J App Biol Biotech. 2017; 5 (01): 079-083. DOI: 10.7324/JABB.2017.50114