Journal of Applied Biology & Biotechnology Vol. 5 (01), pp. 079-083, Jan-Feb, 2017
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2017.50114

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

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ARTICLE INFO

Article history:
Received on: 15/09/2016
Revised on: 30/09/2016
Accepted on: 17/10/2016
Available online: 20/01/2017

Key words:
Oxalate Descarboxilase; Necrotrophic; Genetic Transformation.

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 secrets 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^{2+} 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].

Guimarães and Stotz (2004) [8] showed that OA changes osmoregulation of guard cells inhibiting stomatal closing which leads to wilting and leaf necrosis. Kim et al. (2008) [9] showed that OA triggers apoptosis in plants by a mechanism involving reactive oxygen species (ROS). Using *Sclerotinia sclerotiorum* mutated for oxalate descarboxilase 1 and 2 [10] demonstrated that OA accumulation is developmentally regulated and its activity is required for penetration-dependent infection on many hosts.

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].

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Tobacco, tomato, soybean and lettuce transformed with *OxDc* gene showed tolerance to phytopathogen *Sclerotinia sclerotiorum* [13, 14, 15] and to *Moniliophtora 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

2.1Tobacco transformation

Agrobacterium GV3101 carrying pCambiaOxDc was grown in Lysogny 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 35S CaMV promoter and the selectable marker gene *htp* that confer hygromycin tolerance (Figure 1).

2.2PCR screening of transformed plants

Plantlets of both T_0 and T_1 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'-TCCGGAAGTGCTTGACATTGG-3') and hyg 672 (5'-ATGTTGGCGACCTCGGTATTGG-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.

2.3Progeny analysis

Seeds of the first generation (T_1) 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 χ^2 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.

2.4Transgenic lineages resistance test against oxalic acid

Both T_0 and T_1 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.

2.5Transgenic lineages resistance test against *F. oxysporum*

Both T_0 and T_1 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 line for T_0 and three leaves from each transgenic and nontransgenic for T_1). 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].

2.6RT-PCR expression analysis

Transgenic lines (T_0 and T_1 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´-GAGCTAATACGTGCAACAAACC-3´ and 5´ AGGGAATTGCTCCTAGGTAA-3′).

Fig. 1: Expression cassete T-DNA from pCambia1390 containing *Flamulina velutipes* Oxalate Descarboxilase 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

3.1Transgenic T_0 analysis

3.1.1Tolerance to oxalic acid in T_0 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.

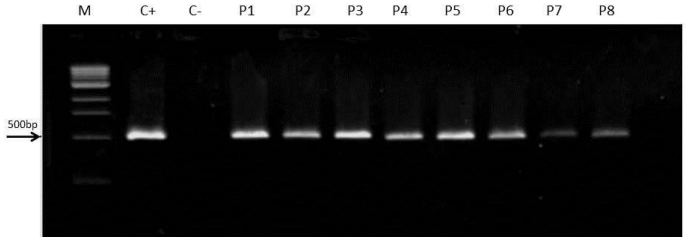

Fig. 2: PCR of transgenic lineages of *Nicotiana tabacum* cv xanthi carrying *OxDc* gene. M (molecular marker 1 kb ladder); C+ (positive control –plasmid pCambiaoxdc); C - (negative control – water); P1 - P8 (transgenic lineages).

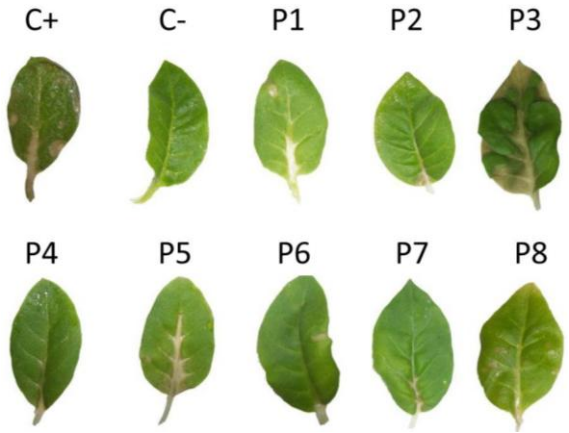

Fig. 3: Treatment of Oxalic acid solution 20 mM OA (pH 4.0) after 48 hours. C+ (positive control – WT plant); C-(negative control – acetic acid solution 20 mM pH4.0); P1-P8 (transgenic lineages).

3.1.2Tolerance of T_0 transgenic lineages to Fusarium oxysporum

Detached leaves of transgenic plants were inoculated with a 1 cm^2 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].

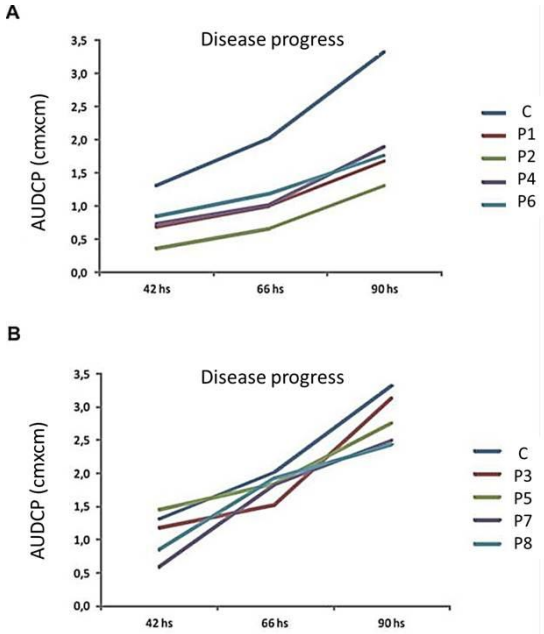

Fig. 4: Disease Progression of transgenic lineages leafs infected with *Fusarium oxysporum.* A) Time progression of area under the disease progress curve (AUDCP); B) C (control), P1, P2, P4 e P6; B: C (control), P3, P5, P7 e P8.

3.1.3 Expression analysis of OxDc in T_0 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).

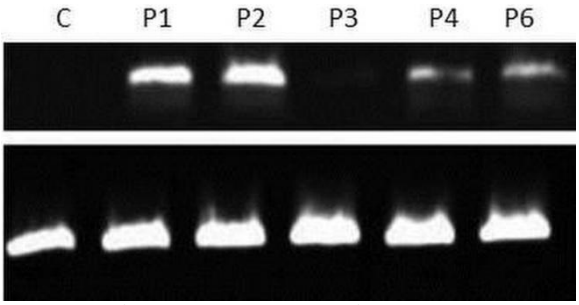

Fig. 5: RT- PCR of transgenic lineages T0 of *Nicotiana tabacum* cv xanthi carrying *OxDc* gene. C - (negative control – water); P1 - P8 (transgenic lineages).

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.

3.2Transgenic T_1 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 specifics 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.

Lineages T_0	Generation T_1*		Segregation ratio tested	χ^2
	Positive	Negative		
P1	25	5	3:1	1,11
P2	24	6	3:1	0,4
P4	25	5	3:1	1,11
P6	22	8	3:1	0,04

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

3.2.1Resistance to oxalic acid in T_1 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.

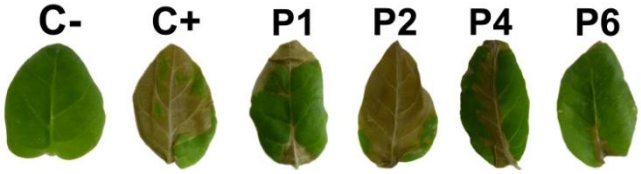

Fig. 6: Treatment of Oxalic acid solution 20 mM OA (pH 4.0) after 48 hours. C+ (positive control – WT plant); C-(negative control – acetic acid solution 20 mM pH4.0); P1, P2, P4 and P6 (transgenic lineages according to T0 AUDCP).

3.2.2Resistance test of T_1 transgenic lineages to Fusarium oxysporum

Detached leaves of transgenic T_1 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 T_0, 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.

3.2.3RT-PCR OxDc expression analysis in T_1 transgenic lineages

Finally, RT- PCR analysis detected the expression of *OxDc* in T_1 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 T_0 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 T_0 plants, T_1 showed similar results, confirming that lower expression levels of *OxDc* gene is directly related to tolerance symptoms.

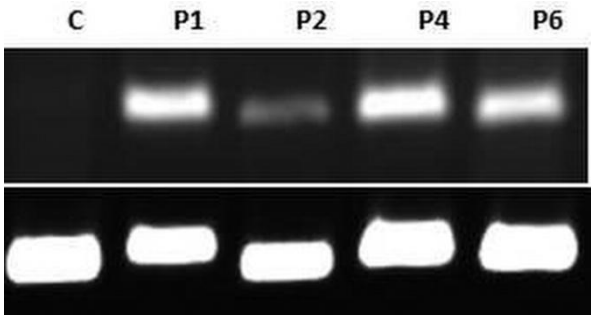

Fig. 7: RT- PCR of transgenic lineages T1 of *Nicotiana tabacum* cv xanthi carrying *OxDc* gene. C - (negative control – water); P1, P2, P4 and P6 (transgenic resistant lineages according to T0 AUDCP).

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 descarboxilase 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

1. Grover A, Gowthaman R. Strategies for development of fungus-resistant transgenic plants. Current Science. 2003, 84: 330–340.
2. García-Guzmán, G, Heil M. Life histories of hosts and pathogens predict patterns in tropical fungal plant diseases. New Phytologist. 2014, 201:1106-1120.
3. Koike ST, Gordon TR. Management of *Fusarium* wilt of strawberry. Crop Protection. 2015, 73:67-72.
4. Pietro AD, Madrid MP, Caracuel Z, Delgado-Jarana J, Roncero MIG. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology. 2003, 4(5):315-325.
5. Favaron F, Sella L, D'ovidio R. Relationships among endopolygalacturonase, oxalate, pH, and plant polygalacturonase-inhibiting protein (PGIP) in the interationc between *Sclerotinia sclerotiorum* and soybean. Molecular Plant Microbe Interactions. 2004, 17:1402-1409.
6. Cessna SG, Sears VE, Dickman MB, Low PS. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. The Plant Cell Online. 2000; 12(11):2191-2199.
7. Dutton MV, Evans, CS. Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology. 1996, 42:881-895.
8. Guimarães RL, Stotz H. Oxalate production by Sclerotinia sclerotiorum deregulates guard cells during infection. Plant physiology. 2004,136(3):3703-3711.
9. Kim KS, Min JY, Dickman MB. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. Molecular Plant-Microbe Interactions. 2008, 21 (5):605-612.
10. Liang X, Moomaw EW, Rollins JA. Fungal oxalate decarboxylase activity contributes to *Sclerotinia sclerotiorum* early infection by affecting both compound appressoria development and function. Molecular Plant Pathology. 2015, 16(8):825-836.
11. Silva LF, Dias CV, Cidade LC, Mendes JS, Pirovani CP, Alvim FC, Pereira GAG, Aragão FJL, Cascardo JCM, Costa MG. Expression of an oxalate decarboxylase impairs the necrotic effect induced by Nep1 -like protein (NLP) of *Moniliophthora perniciosa* in transgenic tobacco. Molecular Plant-Microbe Interactions. 2011, 24:839-848.
12. Mehta A, Datta A. Oxalate decarboxylase from *Collybia velutipes*: Purification, characterization and cDNA cloning. Journal of Biological Chemistry. 1991, 266:23548–23553.
13. Kesarwani M, Azam K, Natarajan A, Mehta A, Datta A. Oxalate Decarboxylase from *Collybia velutipes*Molecular cloning and its overexpression to confer resistance to fungal infection in transgenic tobacco and tomato. Journal of Biological Chemistry. 2000, 275:7230–7238.
14. Dias BBA, Cunha WG, Morais LS, Vianna GR, Rech EL, Capdeville GD, Aragão FJL. Expression of an oxalate decarboxylase gene from *Flammulina* spin transgenic lettuce (*Lactuca sativa*) plants and resistance to *Sclerotinia sclerotiorum.* Plant Pathology. 2006, 55:187-193.
15. Cunha WG, Tinoco MLP, Pancoti HL, Ribeiro RE, Aragão FJL. High resistance to *Sclerotinia sclerotiorum* in transgenic soybean plants transformed to express an oxalate decarboxylase gene. Plant Pathology 2010; 59(4):654-660.
16. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 1962, 15:473–497.
17. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin. 1987, 19:11–15.
18. Dickson MH, Hunter JE. Modification of methods for screening for white mold resistance in greenhouse. Annual Report of the Bean Improvement Cooperative. 1983, 26:85–86.
19. Shaner G, Finney RE. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 1977, 67: 1051–1056.
20. Weinhold A, Kallenbach M, Baldwin IT. Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic Nicotiana attenuata plants. BMC Plant Biology, 2013, 13:99.
21. Swarupa V, Ravishankar KV, Rekha A. Plant defense response against *Fusarium oxysporum* and strategies to develop tolerant genotypes in banana. Planta. 2014, 239(4):735-751.
22. Liu Z, Luan Y, Li J, Yin Y. Expression of a tomato MYB gene in transgenic tobacco increases resistance to *Fusarium oxysporum* and *Botrytis cinerea*. European Journal of Plant Pathology, 2015, 144(3):607-617.

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 descarboxilase gene. J App Biol Biotech. 2017; 5 (01): 079-083. DOI: 10.7324/JABB.2017.50114