SCIENTIFIC NOTE

Colonization of Rice and *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) Larvae by Genetically Modified Endophytic *Methylobacterium mesophilicum*

Fátima T Rampelotti-Ferreira[1], Anderson Ferreira[2], José D Vendramim[1], Paulo T Lacava[2], João L Azevedo[2], Welington L Araújo[2,3]

[1]*Depto de Entomologia e Acarologia, ESALQ, USP, CP 09, 13.418-900 Piracicaba SP, Brasil*
[2]*Depto de Genética, ESALQ, USP, CP 83, 13.400-970 Piracicaba, SP, Brasil*
[3]*Núcleo Integrado de Biotecnologia, Univ de Mogi das Cruzes, Av Dr. Cândido Xavier de Almeida Souza 200, 08.780-911, Mogi das Cruzes, SP, Brasil*

Edited by Fernando L Cônsoli – ESALQ/USP

Neotropical Entomology 39(2):308-310 (2010)

ABSTRACT - The colonization of *Spodoptera frugiperda* J.E. Smith larvae and rice seedlings by genetically modified endophytic bacterium *Methylobacterium mesophilicum,* and also the possible transfer of this bacterium to inside the larva's body during seedlings consumption were studied. The data obtained by bacterial reisolation and fluorescence microscopy showed that the bacterium colonized the rice seedlings, the larva's body and that the endophytic bacteria present in seedlings could be acquired by the larvae. In that way, the transference of endophytic bacterium from plants to insect can be a new and important strategy to insect control using engineered microorganisms.

KEY WORDS: Insecta, *Oryza sativa*, endophytic microorganism

The biotechnological progresses are allowing the identification of endophytic microorganisms promising for insect biological control (Azevedo *et al* 2000). Azevedo & Araújo (2007) defined endophytes as all the microorganisms that inhabit the interior of the host plant, without causing apparent damages or visible external structure. The presence of endophytic microorganisms inside the host plant may increase the plant fitness by protecting it against pest and pathogens, improving plant growth and increasing resistance in stressful environments (Azevedo *et al* 2000, Scherwinski *et al* 2007). Besides the natural occurrence of endophytes, many studies are being carried out with genetically modified microorganisms (GMM) to evaluate host colonization (Germaine *et al* 2004, Ferreira *et al* 2008). *Methylobacterium* spp. have been described as related with plant systemic resistance (Madhaiyan *et al* 2004), plant growth and root formation (Senthilkumar *et al* 2009). In this context, the aim of this work was to study the endophytic colonization of rice seedlings and *Spodoptera frugiperda* J.E. Smith larvae by the genetically modified *Methylobacterium mesophilicum* in *in vitro* conditions.

The endophyte *M. mesophilicum* strain SR1.6/6 used in this work was previously isolated from *Citrus sinensis* (Araújo *et al* 2002) and labeled with Green Fluorescent Protein (*gfp*) (Gai *et al* 2009). The *M. mesophilicum* was introduced in rice seeds to evaluate the plant colonization (Experiment 1), using rice seeds (SCS BRS 112) germinated on MS culture medium (Murashige & Skoog 1962). Seven days after germination, the seedlings roots were submerged in a bacterial cells suspension (10^8 cells ml^{-1}) for 1h and transplanted onto MS medium. Bacterial reisolation was carried out five and ten days after inoculation. Five seedlings were submitted to surface disinfection using serial washing in 70% ethanol for 1 min, 2% sodium hypochlorite solution for 2 min, 70% ethanol for 1 min, and two washes in sterilized distilled water. After surface disinfection, the aerial part of these seedlings was triturated in sterile phosphate buffered saline (PBS) and appropriated dilutions were plated onto CHOI 3 (Toyama *et al* 1998) culture medium amended with 50 µg ml^{-1} of tetracycline antibiotic. Plates were incubated at 28°C for 10 days, upon which the numbers of colony-forming units (CFU) were counted. The disinfection process was checked by plating aliquots of the sterile distilled water used in the final wash onto CHOI 3 and incubated in the same conditions. Also, seedling colonization was evaluated by fluorescence microscopy.

Later, the *M. mesophilicum* interaction with *S. frugiperda* was studied (Experiment 2), where third instars of *S. frugiperda* were maintained in laboratory under artificial diet (Parra 2001, Ferreira Filho pers com). For this, *M. mesophilicum* cells were inoculated onto the diet by aspersion of 50 µl of bacterial suspension (10^8 cells ml^{-1}) and one larva was transferred to each tube, and maintained in laboratory conditions. The control was done using non-inoculated diet

tubes. The experiment was done twice, using five replicates per experiment. The analysis to detect the presence of the *M. mesophilicum* inside the entire larva's body were carried out after 24, 48 e 96h of feeding. Larvae were surface disinfected and bacterial isolation was carried out as described for the rice seedlings. However, the larvae heads were separate from the body, and triturated in PBS. The fecal pellets were also diluted in PBS and plated onto CHOI 3 medium to evaluate the presence of *gfp* expressing *M. mesophilicum*. Afterwards, we studied the possible transference of *M. mesophilicum* from rice seedlings to *S. frugiperda* during the larva feeding (Experiment 3). Third instars of *S. frugiperda* were used in this bioassay. Rice seedlings were inoculated with *M. mesophilicum* as early described in experiment 1 and were maintained in laboratory conditions to one week, when the aerial part of these seedlings was introduced into the tubes containing one *S. frugiperda* larva. The presence of endophytic *M. mesophilicum* associated to inner larvae parts, such as heads, body and feces were carried out after 96h.

The *gfp* tagged endophytic *M. mesophilicum* colonized rice seedlings after root inoculation. This colonization was observed by reisolation and fluorescence microscopy analysis. The bacterial density ranged from 5×10^1 to 8.2×10^1 CFU.g^{-1} of rice tissue five and ten days after inoculation, respectively.

Also, 96h after inoculation of the *gfp* tagged *M. mesophilicum* onto diet, the bacterium was found inside the larvae, suggesting that the larvae fed the diet with the tagged bacterium, which survived inside the insect. The bacterial density inside the larval body ranged from 2×10^2 to 8×10^2 CFU.g^{-1}. *Methylobacterium mesophilicum* was not observed in feces samples.

The bacterium inoculated by the roots was able to colonize rice seedlings and be further transferred from the plant to the larvae during insect feeding, reaching 10×10^1 CFU.g^{-1} of surface disinfected larval tissues. *Methylobacterium mesophilicum* was not observed in the heads and feces samples, suggesting that although this bacterium could settle inside the larvae, it was not release with the feces. Also, this result indicates that this strategy could be used for biological control of insect that feed on important economical crops, since the *gfp* gene could be changed by other genes, such as Bt*cry* or protease inhibitors. Similar model was previous proposed by Fahey *et al* (1991) and Tomasino *et al* (1995), which used endophytic bacteria carrying the Bt *cry* genes to protect plants against insect attack.

However, more studies on the mechanisms involved in insect, plant and bacterium interaction are necessary to define a better control strategy. Although, some authors have described *M. mesophilicum* as a cause of opportunistic infections in immunocompromised hosts (Sanders *et al* 2000, Imbert *et al* 2005), many others described the biotechnological importance of this bacterium (Holland 1997, Madhayan *et al* 2004, Senthilkumar *et al* 2009), suggesting that diversity and applied studies should be carried out together, to choose some strains able to improve plant protection with low risks to human healthy. The transference of endophytic bacterium from plants to insect can be a new and important strategy to insect control using engineered microorganisms able to express toxins inside the plant and/or insect.

Acknowledgments

We thank Mateus Mondin and Dr Margarida L R de Aguiar-Perecin of the Laboratory of Molecular Cytogenetics for the use of the fluorescent microscope facilities and the support of the CNPq (National Council of Research, Brazil).

References

Araújo W L, Marcon J, Maccheroni J, Elsas J D V, Vuurde J W L V, Azevedo J L (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl Environ Microbiol 68: 4906-4914.

Arthur V, Aguilar J A D, Arthur P B (2002) Esterilização de adultos de *Spodoptera frugiperda* a partir de pupas irradiadas. Arq Inst Biol 69: 75-77.

Azevedo J L, Araújo W L (2007) Diversity and applications of endophytic fungi isolated from tropical plants, p.189-207. In Ganguli B N, Deshmukh S K (eds) Fungi: multifaceted microbes. Boca Raton, CRC press, 321p.

Azevedo J L, Macheroni Jr W, Pereira J O, Araújo W L de (2000) Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron J Biotechnol 3: 40-65.

Fahey J W, Dimock M B, Tomasino S F, Taylor J M, Carlson P S (1991) Genetically engineered endophytes as biocontrol agents: a case study in industry, p.401-411. In Andrews J H, Hirano S S (eds) Microbial ecology of leaves. New York, Springer, Verlag, 499p.

Gai C S, Lacava P T, Quecine M C, Auriac M C, Lopes J R S, Araújo W L, Miller T A, Azevedo J L (2009) Transmission of *Methylobacterium mesophilicum* by *Bucephalogonia xanthophis* for paratransgenic control strategy of Citrus Variegated Chlorosis. J Microbiol 47: 448-454.

Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, van Der Lelie D, Barac T, Oeyen L, Vangronsveld J, Moore F P, Moore E R B, Campbell C D, Ryan D, Dowling D N (2004) Colonisation of poplar trees by *gfp* expressing bacterial endophytes. FEMS Microbiol Ecol 48: 109-118.

Holland M A (1997) *Methylobacterium* in plants. Recent Res Dev Plant Physiol 1: 207-213.

Imbert G, Seccia Y, La Scola B (2005) *Methylobacterium* sp. bacteraemia due to a contaminated endoscope. J Hosp Infec 61: 268-270.

Madhaiyan M, Poonguzhali S, Ryu J, As T (2004) Growth promotion and induction of systemic resistance in rice cultivar Co-47 (*Oryza sativa* L.) by *Methylobacterium* spp. Bot Bull Acad Sin 45: 315-324.

Parra J R P (2001) Técnicas de criação de insetos para programas de controle biológico. Piracicaba, ESALQ/FEALQ, 6 ed, 134p.

Sanders J W, Martin J W, Hooke M, Hooke J (2000) Methylobacterium mesophilicum infection: case report and literature review of an unusual opportunistic pathogen. Clin Infect Dis 30: 936-938.

Scherwinski K, Wolf A, Berg G (2007) Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 as model bactéria. Bio Control 52: 87-112.

Senthilkumar M, Madhaiyan M, Sundaram S P, Kannaiyan S (2009) Intercellular colonization and growth promoting effects of *Methylobacterium* sp. with plant-growth regulators on rice (*Oryza sativa* L. Cv CO-43). Microbiol Res 164: 92-104.

Tomasino S F, Leister R T, Dimock M B, Beach R M, Kelly J L (1995) Field performance of *Clavibacter xyli* subsp. *cynodontis* expressing the insecticidal protein gene *cryIA*(*c*) of *Bacillus thuringiensis* against European corn borer in field corn. Biol Control 5: 442-448.

Toyama H, Anthony C, Lidstrom M E (1998) Construction of insertion and deletion mxa mutants of *Methylobacterium extorquens* AM1 by electroporation. FEMS Microbiol Lett 166: 1-7.

Received 31/VII/08. Accepted 15/IX/09.