In vivo Efficacy of Lactic Acid Bacteria in Biological Control against *Fusarium oxysporum* for Protection of Tomato Plant

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Abstract: Lactic acid bacteria (LAB), isolated from milk and yoghurt, were tested for their efficacy against some phytopathogenic fungi under *in vitro* and *in vivo* tests. *Fusarium oxysporum*, one of most important pathogenic fungi invade tomato plants, was chosen to evaluate the effectiveness of LAB as a biocontrol agent under *in vivo* tests. Culture broth of LAB was applied as seed treatment or soil drench. The protective effect of LAB significantly increased after challenging inoculation by *F. oxysporem*, especially when LAB were applied as seed treatment; the number of roots increased by 216, 311, and 358% over control with LB-1, LB-4, and LB-5, respectively, whereas the increment was 169, 163, and 181% for soil drench. Interestingly, when LAB were applied as seed treatment, in soil infested with *F.oxysporum*, the total fresh weight of tomato plants increased by 348, 260, and 390% with LB-1, LB-3, LB-5, respectively, whereas the increment was 268, 427, and 393% with LB-1, LB-4, and LB-5, respectively, for soil drench. Overall, while previous reports of antifungal activity by LAB under *in vitro* tests are scarce, we have demonstrated for the first time the capability of LAB to act as plant growth promoting bacteria and biocontrol agent against some phytopathogenic fungi under *in vivo* tests.

[Hoda A. Hamed, Yomna A. Moustafa and Shadia M. Abdel-Aziz. *In vivo* Efficacy of Lactic Acid Bacteria in Biological Control against *Fusarium oxysporum* for Protection of Tomato Plant**]** Life Science Journal,. 2011; 8(4): 462-468] (ISSN: 1097-8135). http://www.lifesciencesite.com. 60

Keywords: Lactic acid bacteria, biocontrol, plant growth promoting, plant pathogenic fungi, *Fusarium oxysporum*.

1. Introduction

Soil-borne fungal diseases are among the most important factors limiting the yield of many economically important plants, resulting in serious economic losses. Several soil-pathogens attack roots and shoots of plants, causing damping-off or root-rot (**Elad *et al.*, 1982; Thomasho, 1996**). Chemical fertilizers and pesticides are extensively used to prevent or control plant diseases. However, the environmental pollution caused by excessive use of such agrochemicals as well as the development of resistant pathogens have promoted the search for alternative approaches, i.e. the use of microorganisms or their metabolites (**Ronel *et al.*, 1986; Montesinos, 2003**). It has been established that different species of bacteria and fungi such as *Trichoderma*, *Pseudomonas*, *Stryptomyces*, and *Bacillus* are used as antimicrobial agents against phytopathogenic fungi. Recently, LAB have received much attention. Application of LAB in traditional food and feed fermentation and preservation is well documented (**Kim, 1993; Johan and Jesper, 2005**). LAB are harmless and used to improve human and animal health (probiotics). LAB have a GRAS state (generally recognized as safe) and it has been estimated that 25% of the European diet and 60% of the diet in many developing countries consist of fermented foods (**Stiles, 1996**). LAB produce a variety of antimicrobial compounds and effective substances such as lactic, acetic, probionic acids, antibiotics, bacteriocins as well as hydrogen peroxide and carbon dioxide (**Ouwehand, 1998**). The precise mechanism of antimicrobial action is difficult to elucidate due to the complex and commonly synergistic interactions between different compounds (**Corsetti *et al.*, 1998**). However, the mechanism can be attributed to both competition for nutrient and production of antibiotics and various inhibitory substances (**Johan and Jesper, 2005**). Many of the antimicrobial compounds affect the physiological activities of a pathogen such as cell division, biosynthesis of DNA, RNA, protein, lipid metabolism and cell synthesis (**Chaurasia *et al.*, 2005**). The effect of antibiotics and other compounds produced by LAB has widely been researched, especially in fermented foods (**Johen and Jesper, 2005**; **Lindgren and Dobrogosz, 1990),** silage (**Stirling and Whittenbury, 1963; Woolford, 1994**), and as biopreservatives (**Johan and Jesper, 2005**).

During the past 50 years, many studies were reported about bacterial and fungal plant diseases as well as the application of different microorganisms as biocontrol agents. However, no information is available on the interactions of LAB with phytopathogenic fungi (**Stephane *et al.*, 2005; Ashgar and Mohammad, 2010**). Very few *in vitro* studies have been reported about the efficacy of LAB

against phytopathogenic fungi (**Zulpa *et al.*, 2003; Rosalia *et al.*, 2008; Wang *et al.*, 2011**).

The aim of our contribution was to: 1) evaluate the influence of some strains of lactobacilli against some phytopathogenic fungi under *in vitro* tests , 2) Assess the efficacy of LAB to act as plant growth-promoting bacteria (PGPB) for tomato plants under *in vivo* tests, and 3) evaluate the potential of LAB against *F. oxysporum* in pot trials, using culture broth of LAB applied as seed treatment or soil drench. To the best of our knowledge, this is the first study dealt with the potential of LAB against the fungus *F. oxysporum* under *in vivo* tests.

2. Material and Methods

Lactic acid bacteria for antifungal activity

Lactic acid bacteria (LAB), included *Lactobacillus* sp. 1 (LB-1), *Lactobacillus acidophilus* sp. 2 (LB-2), *Lactobacillus* sp.4 (LB-4), *Lactobacillus* sp.5 (LB-5), were previously isolated from yoghurt and milk. In addition, a lyophilized strain of *Lactobacillus plantarum* NRRL B-4524 (LB-3) was from the National Center for Agricultural Utilization Research (USA). All strains were kept on MRS agar (Van den Berg *et al.*, 1993). Fresh cultures are grown in MRS broth at 30^{o}C for 24hrs before use in experiments.

Phytopathogenic fungi

High virulent strains of pathogenic fungi, previously isolated from diseased plants, were used in this study. *Fusarium oxysporum*-1 (isolated from tomato), *Rhizoctonia solani*-1 (isolated from lupine), *F.oxysporum*-2 (isolated from cotton), *Rhizoctonia solani*-2 (isolated from tomato), and *Sclorotium rolfsii* (isolated from onion). Fungal strains were maintained on potato dextrose agar (PDA) at 4^{o}C.

Preparation of spore suspension

F. oxysporum-1 was grown in PDB at 28^{o}C. After incubation for 7 days, fungal biomass was homogenized in a blender for one minute. Spore suspensions were prepared using sterile distilled water to a concentration of 5x10^{5} spores. The spore suspension was prepared just before each experiment.

In vitro assays

In vitro assays for antifungal activity by LAB were determined using conical flasks (250-ml) containing PDB as the growth medium for all test fungi. Flasks, supplemented with LAB, were inoculated with the test fungi and incubated at 28^{o}C for each of all fungal strains. After incubation for 7days, fungal growth were filtered, washed several times with distilled water, and dried at 55^{o}C to a constant weight. Percentage of growth inhibition (GI) was calculated using the formula: GI (%) = $C_0 - C_F / C_o$ x 100%, where C_0 is the dry weight of fungal mycelium (control), C_F is the dry weight of fungal mycelium after inhibition by LAB.

In vivo experimental design

Preparation of Tomato seedlings

Peat moss soil was dispensed into plastic trays (160 eyes) for growth of seedlings. Moisture content of peat moss was sustained at a proper level throughout seedlings growth. Tomato seeds (UC 97) were divided into two groups: in one group, seeds were soaked in culture broth of LAB strains for 1 hr; 1ml for one seed was applied. The second group included non-soaked seeds. After 45 days, healthy seedlings were transplanted for pot trials.

In pot trials

All experiments were performed in pots, 30 cm diameter, filled with unsterilized natural soil. Uninoculated or inoculated soil with *F.oxysporum*-1, at a rate of 10-ml homogenized culture per pot, were prepared one day before planting. Two treatments were performed: firstly, in which tomato seeds were pre-soaked in culture broth of LAB, resultant seedlings were placed in pots uninoculated or inoculated with *Fusarium*, without supplementation of soil with LAB (seed treatment). Secondly, where non-soaked seeds were used, seedlings were also placed in pots in absence and presence of *Fusarium* with supplementation of soil with LAB, at a rate of 10-ml culture broth per pot (soil drench). All experiments and controls were replicated three times. Plants were drenching at intervals and grown for another 45 days. *In vivo* tests were performed during 3-months, from April up to June, where the atmospheric temperature ranged from 25 - 38^{o}C.

Sampling and analysis

After three months growth, tomato plants were harvested. To reveal the effect of LAB on the growth characteristics, each plant was measured for shoot- and root-length, number of secondary roots, and total fresh weight of plants.

Statistical analysis

Data were analyzed using SPSS for windows (SPSS Inc.) by means of a one-way ANOVA and subsequently differences between treatments were determined using least significant differences (LSD at α 0.05).

3. Results and Discussion

In vitro efficacy of LAB against phytopathogenic fungi

Results of *in vitro* inhibition by LAB revealed that fungal strains behaved differently. Low**,** moderate, to high inhibition effects were observed by LAB against the pathogenic fungi. Low inhibition effect was observed by all strains of LAB against *F.oxysporum*-1, whereas both of *R.solani*-1 and *F.oxysporum*-2 were moderately inhibited by LB-2, LB-3, and LB-4 (Fig. 1). High inhibition effect was observed against *S. rolfsii*; it was highly inhibited by 86, 84, and 75% with LB-2, LB-3, LB-4, respectively. The only exception as negative impact by LAB was observed with LB-1 which showed growth promotion for the fungus *R.solani-2;* the dry weight of the fungal mycelium reached 114*%* over control (inhibition 0%, Fig.1). Thus, *in vitro* studies revealed that *S. rolfsii* was the most inhibited pathogen by LB-2, LB-3 and LB-4, whereas LB-1 and LB-5 showed the lowest inhibition effect against most of the tested pathogens. In addition, *F.oxysporum*-1 was the most resistant fungus to all the strains of LAB under *in vitro* tests. We have found very few references about *in vitro* effect of LAB as biocontrol agents against phytopathogenic fungi. **Zulpa *et al.* (2003)** reported that the inhibitory effect of extracellular products of *Streptococcus thermophilus* against "wood blue stain fungi" could be attributed to antimicrobial substances such as lactic acid ,organic acid ,and hydrogen peroxide. **Rosalia *et al.* (2008)** have reported that, LAB isolated from fresh fruits and vegetables, were found to produce organic acid substances that affected some phytopathogenes, causal of postharvest. **Wang *et al.* (2011)** found that, metabolites of *Lactobacillus plantarum* IMAU10014 possess high activity against some plant pathogenic fungi and that the antifungal effect is attributed to a proteinaceous substance. In our study, growth inhibition of phytopathogenic fungi by LAB using PDB medium indicated that, even in competition with the pathogen under conditions more favorable to the pathogen, reduction in fungal growth was observed.

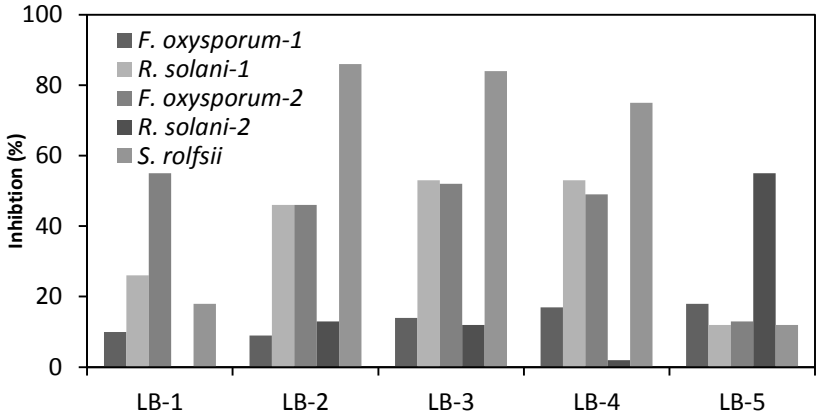

Fig.1. The percentage of growth inhibition of phytopathogenic fungi by lactic acid bacterial strains; LB-1, LB-2, LB-3, LB-4, and LB-5.

The inhibitory effect of LAB may also be attributed to the production of antibiotics, especially nisin (Yomna, 2000) as well as competition for nutrients as indicated above. On other hand, the low inhibition effect by LAB against *F.oxysporum*-1 may be elucidate by an assumption that the concentration of the produced inhibitors may not be high enough to inhibit the fungal growth. The opposite effect (114% growth promotion) occurred by LB-1 with *R. solani*-2 (Fig.1) may be attributed to the growth-promoting effect for a pathogen , i.e. some bacterial strains produce opposite effects (growth promotion or inhibition) depending on the sensitivity threshold of the fungus to the extracellular metabolites produced by bacteria (**Don *et al.*, 1993; Zulpa *et al.*, 2003**).

***In vivo* efficacy by lactic acid bacteria**

Being the most resistant fungus to all strains of LAB under *in vitro* tests, *F.oxysporum*-1 was chosen for *in vivo* tests using tomato seeds.

Plant growth-promoting effect by lactic acid bacteria

In absence of *Fusarium*, results of pot trials with LAB applied as seed treatment or soil drench revealed their ability to enhance plant growth compare with control (Fig. 2).

However, the plant growth characteristics significantly differed in response to LAB strains. When LAB was applied as seed treatment, the shoot length was 28.00 and 37.67cm (112 and 151% over control) with LB-1 and LB-5, respectively, with no obvious increase in root length (Table 1). However,

the number of secondary roots increased by 48, 41, and 62 (171, 146, and 221% over control) with LB-1, LB-3, and LB-5, respectively. On other hand, when LAB were applied as soil drench, the shoot length was 32.50, 34.33, and 35.00 cm (135, 143, and 146% over control) with LB-1, LB-2, and LB-5, respectively (Table 1); whereas, number of secondary roots increased by 36, 35, and 42 (164, 159, and 191% over control) with LB-1, LB-2, and LB-5, respectively (Table 1). These results reveal the capability of LAB to be considered as PGPB. Application of PGPB has been hampered by inconsistent performance in field tests; this is usually attributed to their poor rhizosphere competence (**Thomashow, 1996**). Rhizosphere competence of biological control agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period in presence of the indigenous microflora (**Stephane *et al.*, 2005**).

Fig. 2. Plant growth promoting effect of lactic acid bacteria for tomato plant compare with control, 0 (left image; plant without bacteria or fungi). Culture broth of lactic acid bacteria was applied as seed treatment (middle image) or soil drench (right image). Plants were harvested after 3-months growth.

Table 1. Mean shoot- and root-length (cm), and number of secondary roots per root system of tomato plants, treated with lactic acid bacteria, in soil non-infested with *Fusarium*.

LAB	Seed treatment			Soil drench		
	Shoot length	Root length	S-roots*	Shoot Length	Root length	S-roots*
Control	25.00^{ab}	14.50^{a}	28.00	24.00^{ab}	15.50^{b}	22.00
LB-1	28.00^{f}	14.33^{fg}	48.00	32.50^{de}	19.00^{b}	36.00
LB-2	20.17^{j}	12.67^{hg}	39.00	34.33^{cd}	18.67^{b}	35.00
LB-3	25.33^{g}	15.33^{ef}	41.00	12.17^{ij}	12.67^{gh}	22.00
LB-4	16.83^{k}	11.50^{h}	25.00	27.67^{f}	14.00^{fg}	29.00
LB-5	37.67^{b}	14.33^{fg}	62.00	35.00^{c}	31.00^{a}	42.00

*S-roots = number of secondary roots, one value was chosen from three replicates.

Our results could confirm efficacy of LAB as PGPB; bioprotection of tomato seeds and the soil drench with LAB support plant growth, especially that a single application by LAB was used. It seems that nutrient availability for tomato plants is not limited despite of long duration (3-months of seed treatment or 45 days of soil drench). **Lewis and Papaviza (1984)** reprted that, *Trichoderma harzianum* required a constant nutrient supplementation for optimum biocontrol efficacy against phytopathogenic fungi. **Rose *et al.* (2003)** reported that, two applications of the biocontrol agents *Trichoderma harzianum*, *Pseudomonas chlororaphis* and *Streptomyces griseoviridis* were needed every ten days for effective controlling against *F. oxysporum*.

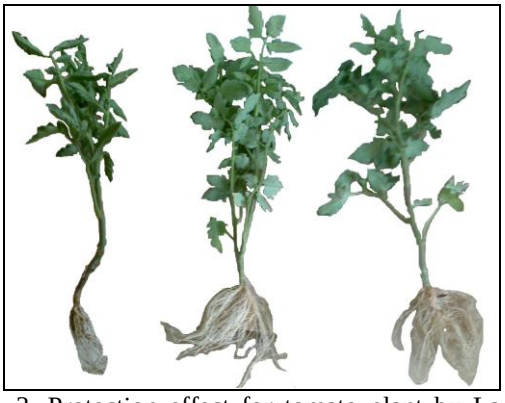

Fig. 3. Protection effect for tomato plant by Lactic acid bacteria as biocontrol agent against *F. oxysporum*-1 (control plant+fungi, left image). Culture broth of lactic acid bacteria was applied as seed treatment (middle image) or soil drench (right image). Plants were harvested after 3-months growth.

Antifungal activity by lactic acid bacteria

LAB could be considered as PGPB as indicated above. The protective effect of LAB significantly increased after challenging inoculation of soil with *F. oxyspoium*-1 (Fig. 3). Moreover, plant measurements and the number of secondary roots significantly increased with all strains of LAB (Table 2). When LAB were applied as seed treatment, the number of secondary roots increased by 41, 59, and 68 (216, 311, and 358% over control) with LB-1, LB-4, and LB-5, respectively (Table 2). When soil drench was applied by LAB, the number of secondary roots was 27, 26, and 29 (169, 163, and 181% over control) with LB-1, LB-4, and LB-5, respectively.

Moreover, the total fresh weight (TFW) of tomato plants increased about 2-4 fold with LAB in soil infested with *Fusarium* (Table, 3). The TFW of tomato plants reached 27.37, 20.50, and 30.67g (348, 260, and 390% over control) with LB-1, LB-3, and LB-5, respectively, for seed treatment, whereas it reached 21.13, 33.60, and 30.90g (268, 427, and 393%) with LB-1, LB-4, and LB-5, respectively, for soil drench. A possible mechanism for increasing plant growth and TFW by LAB may be due to the efficiency in nutrient transfer from soil to the roots and plants as a result of increasing the number of roots and bioprotection of rhizosphere area by LAB.

Another mechanism for increasing plant growth and TFW are caused by the antifungal metabolites (antibiotic) produced by LAB against *Fusarium*, especially when synergistic effects of lytic enzymes of fungal cell wall, produced by other potential antagonists are thought, leading to the enrichment of soil with nutrients.

Table 2. Mean shoot- and root-length (cm), and number of secondary roots per root system of tomato plants, treated with lactic acid bacteria, in soil infested with *Fusarium*.

LAB	Seed treatment			Soil drench		
	Shoot length	Root length	S-roots*	Shoot Length	Root length	S-roots*
Control	20.60^{ab}	11.70^{b}	19.00	21.00^{b}	14.50^{b}	16.00
LB-1	24.00^{gh}	18.67^{b}	41.00	31.33^{e}	18.67^{b}	27.00
LB-2	23.67^{gh}	13.00^{gh}	35.00	27.50^{f}	15.67^{ef}	21.00
LB-3	28.10^{f}	16.50^{de}	38.00	22.67^{hi}	16.67^{cde}	21.00
LB-4	31.00^{e}	15.00^{ef}	59.00	31.67^{e}	18.17^{bcd}	26.00
LB-5	38.50^{b}	15.33^{ef}	68.00	44.50^{a}	18.33^{bc}	29.00

*S-roots = number of secondary roots, one value was chosen from three replicates.

In addition, treatment of soil with LAB may trigger systematic acquired resistance (SAR) which develops when plants successfully activate their defense mechanism, in presence of a pathogen infection, resulting in an enhanced synthesis of plant defense chemicals which support plant growth and fortify plant cell wall strength (**Stephane *et al.*, 2005**). In the present study, the obvious elongation in both shoot- and root-length and the increasing number of secondary roots (Fig. 3) as well as the increment in TFW of tomato plants (Table 3) may confirm the capability of LAB to trigger the SAR of tomato plants and induce the production of growth regulators, stimulants, or hormones by which elongation of plant and increment of TFW are occurred (Figs. 2 and 3). Worthy mention is that, LB-1 and LB-5 showed higher antifungal activity under *in vivo* tests, which is contrary to the results of *in vitro* tests. Actually, *in vivo* tests ensure the efficacy of LAB as biocontrol agent against phytopathogenic fungi, indicating that *in vitro* assays are not fully predictive for the inhibitory action confirmed under *in vivo* tests against a pathogen (**Faina *et al.*, 2007**).

Table 3. Mean total fresh weight (g) of tomato plants as affected by treatment with LAB and infection with *F.oxysporum*-1.

LAB	Seed Treatment		Soil Drench	
	Soi l w/o *Fusarium*	Soil with *Fusarium*	Soil w/o *Fusarium*	Soil with *Fusarium*
LB-1	21.00^{e}	27.37^{c}	20.03^{e}	21.13^{e}
LB-2	8.77^{j}	12.83^{gh}	17.77^{f}	12.27^{hi}
LB-3	21.87^{de}	20.50^{e}	13.67^{gh}	12.37^{ghi}
LB-4	10.67^{ij}	9.53^{j}	14.50^{g}	33.60^{a}
LB-5	34.73^{a}	30.67^{b}	23.60^{d}	30.90^{b}

Total fresh weight of control (plant+fungi) = 7.87^{b}. The control value was set as 100%.

The antifungal effect of LAB under *in vivo* conditions may lead us for thinking about the efficacy of LAB as root colonizer. We can't exclude the possibility that on roots, LAB could attack, colonize, and reduce fungal growth much faster than under *in vitro* tests.

LAB are used for preservation of food and milk products from centuries and acquired the GRAS status. Thus, metabolic products of LAB can be safely used in biocontrol of plant pathogenic fungi.

The use of LAB as biocontrol agent against phytopathogenic fungi presents both challenges and opportunities for management of plant diseases. We demonstrated for the first time the efficacy of LAB as biocontrol agent under *in vivo* conditions. The success of biological control in our tests is surprising compare with the control. LAB may produce a variety of antifungal substances under *in vitro* tests, but under *in vivo* tests the mechanism of antifungal action is difficult to elucidate due to the complex and commonly synergistic interactions between different compounds and different soil microbiota (**Naseby *et al.*, 2000; Johan and Jesper, 2005**). It could also be suggested that, a synergistic effect of LAB with other beneficial microorganisms in soil may provide an almost constant nutrient source for the plants. Other potential antagonists in soil may secrete hydrolytic enzymes that degrade *Fusarium* cell wall and produce exopolysaccharides which may contribute for enrichment of soil with nutrients, leading to the plant healthy. *In vivo* tests, however, showed LAB to be considered as biocontrol agent against *F.oxysporum* despite of stress conditions such as fluctuation in temperature, relative humidity, and a greater variety of competitive microorganisms.

The more practical approach has been used in our study is to elucidate the efficacy of LAB, isolated from milk and yoghurt, for biocontrol against phytopathogenic fungi. LAB with antifungal and antibacterial activity are well documented in food, meat, and milk products as biopreservatives (**Johen and Josper, 2005**), while less attention has been paid to exploit the antifungal activity of LAB for biocontrol of phytopathogenic fungi. When this effect was reported, under *in vitro* assays, it was attributed to the production of indol acetic acids and phenolic substances (**Zulpa *et al.*, 2003**), organic acids (Rosalia et al. 2008), or proteinaceous compounds (**Wang *et al.*, 2011**). The antifungal substances produced by LAB in this study are antibiotics (**Yomna, 2000**). However, competition for nutrient and space for preventing the pathogen to colonize the rhizosphere may also be another mechanism.

Conclusion

Bioprotection of tomato plants by LAB isolated from yoghurt and milk is a finding reinforced not only by their inhibition capacity but also by their persistance in soil under hard conditions. LAB seem to be more resistant to stress conditions such as fluctuation in temperature and relative humidity. Furthermore, each strain of LAB showed antifungal activity towards more than one pathogen, under *in vitro* tests. There was no distinct correlation between the *in vitro* positive antagonism and the *in vivo* positive antagonism. The use of chemicals and fungicides in agriculture as well as the environmental pollution would be avoided by LAB as a promising PGPB and biocontrol agent. Future research should confirm the mechanism of inhibition, assay for lytic enzymes, and determination of inhibitor substances other than antibiotics for application of LAB in biocontrol as a viable alternative method to manage plant diseases.

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10/25/2011