1 ***Short Research Article***

# 2 Induction of resistance in poplar to *Melampsora* 3 *larici-populina* using 4 L-form bacteria

5


## 6 Abstract

7 Poplars (*Populus* spp.) of the Family Salicaceae are extensively cultivated worldwide and are 8 susceptible to a variety of bacterial and fungal diseases. In *Populus* species, leaf rust disease 9 caused by several species of *Melampsora* leads to considerable damages in plantations. 10 *Melampsora larici-populina* is the most devastating and widespread fungal pathogen causing 11 leaf rust disease in poplars. In this study, leaves and young stems of rooted cuttings of two 12 poplar clones were treated with L-form bacteria of *Bacillus subtilis* NCIMB 8054, ATCC 13 6633 and then challenged with the spores of rust pathogen *M. larici-populina,*. The 14 development of uredinia was evaluated in the laboratory using the leaf disc assay. The L- 15 forms greatly reduced rust severity in inoculated poplar leaves (local effect), while to a lesser 16 extent in non-inoculated leaves obtained from inoculated plants showing a low systemic 17 effect on pustule development. This plant- L-form symbiosis may have contributed 18 significantly to a quantitative resistance to *M. larici-populina* indicating a promising 19 implication for the use of L-form bacteria of *B. subtilis* as a biocontrol agent for poplars 20 against the rust pathogen.


21


22 **Key words –** L-form bacteria, Plant-L-form association, Induced resistance, *Populus* spp., 23 *Melampsora larici-populina*, Leaf disc assay.


24

## 25 1. Introduction

26 Plants, like humans and other animals, become diseased and these diseases are caused by 27 different animate and inanimate agents. Different approaches are used to prevent, mitigate or 28 control plant diseases (Pal and Gardener, 2006). Conventional disease control is based on the 29 application of various chemicals and resistance breeding. The development of highly 30 effective pesticides seem to offer instant solutions to the threat of disease, but the 31 environmental pollution caused by excessive use and misuse of agrochemicals has changed

32 people's attitudes towards their use. Induced disease resistance is an interesting alternative
33 for the plant protection, which is based on the activation of existing resistance mechanisms in
34 the plants and it is effective against a broad spectrum of plant pathogens (Van Loon *et al*.,
35 1998).

36 Induced resistance in plants can be local or systemic. At least two forms of induced
37 resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) have
38 been characterized as two distinct phenomena based on the types of inducing agents and the
39 signalling pathways of the host that result in resistance expression (Sticher *et al*., 1997, Van
40 Loon *et al*., 1998). Expression of localized necrosis caused by the inducing pathogen is the
41 major characteristic of SAR. This necrosis can be either a hypersensitive response (HR) or a
42 local necrotic lesion caused by the virulent pathogen. SAR is also dependent on salicylic acid
43 signalling and expression of genes of pathogenesis-related proteins (PR proteins) (Sticher *et*
44 *al*., 1997, Hammerschmidt, 1999). ISR is induced by certain strains of plant growth
45 promoting rhizobacteria (PGPR). Unlike SAR, ISR is not associated with necrosis. ISR
46 depends on the perception of ethylene and jasmonic acid and it is not associated with
47 expression of genes for PR proteins (Van Loon *et al*., 1998).

48 L-form bacteria have modified or no cell walls (Madoff, 1986) and are capable of
49 forming non-pathogenic symbioses with a wide range of plants (Paton, 1987). This type of
50 symbiosis confers resistance against subsequent challenge by other fungal and bacterial
51 pathogens (Walker *et al*., 2002, Daulagala and Allan, 2003) and hence the association has the
52 potential as a novel system for biological control. However, the mechanisms for the
53 protection are not well known. But Daulagala and Allan (2003) detected a higher activity of
54 chitinase, a major PR protein of L-form treated Chinese cabbage plants than that of the
55 control plants treated with 5% (w/v) mannitol and this suggested that L-forms have induced
56 the activity of chitinases in plants. In the detached leaf and the whole plant bioassays, the L-
57 form treated Chinese cabbage plants, challenged with *Botrytis cinerea*, consistently showed
58 lower grey mould disease indices than seedlings treated only with mannitol. Furthermore, the
59 resistance expressed by the L-forms was similar to the resistance observed in plants colonized
60 with the mutants of *Colletotrichum magna*, which were no longer pathogenic, but very
61 successfully colonized a wide range of host plants as endophytes (Redman *et al*., 1999). As it
62 is not still clear whether this resistance in plants is an SAR type, it is interesting to study how
63 these non-pathogenic microbes like L-form bacteria trigger defence responses in plants.
64

65 Poplar is a deciduous tree that belongs to the Family Salicaceae. There are about 35 species
66 of poplar trees. This tree grows mostly in temperate climates. Poplar trees grow quickly and
67 provide enough shade. Wood can be used for numerous purposes such as for the production
68 of plywood, musical instruments like guitars, drums and often used in paper industry.
69 Poplars are susceptible for a variety of bacterial and fungal diseases. Rust caused by
70 *Melampsora* sp. is one of the most serious diseases of poplars. Among the *Melampsora*
71 species, *M. larici-populina* is the most widespread and frequent rust species described in
72 poplar and the principal rust fungus concern in Britain. *M. larici-populina* is an obligate
73 macrocyclic basidiomycete, which has its sexual stage on larch (*Larix* sp.) and its asexual
74 stage on poplar. Poplar rust is easily recognized by the masses of yellow/orange fungal spores
75 that cover the under the surface of the leaves. After a few weeks, the leaves blacken, curl up
76 and fall prematurely. Apart from a reduction of growth due to foliage loss, sometimes there is
77 a failure of shoot maturation. Imperfect maturation can lead to a dieback of the shoot, even to
78 the extent that the entire plant may die.
79 There is no pesticide approved for use against poplar rust in woodlands in the UK.
80 Trials elsewhere in Europe have indicated that economically acceptable but partial control of
81 the disease can be achieved by one or two annual applications of a fungicide with curative
82 and persistent properties. However, chemical control of plant diseases is expensive,
83 sometimes physically impracticable and in many cases environmentally undesirable. Use of
84 resistant clones is one of the best disease-control strategies; however, the number of highly
85 resistant clones is limited, making biological control an attractive disease-control alternative.
86 Therefore, there is no doubt that in the future, disease control in plants, presently provided by
87 chemicals, mainly by fungicides and bactericides, will be replaced by new disease control
88 technologies emerging from the knowledge of plant-microbe interactions. This present study
89 was conducted to determine if *M. larici-populina* in poplars could be controlled using L-form
90 bacteria of *B. subtilis* as a safe and alternative strategy to reduce the dependency on synthetic
91 fungicides.
92
93 ## 2. Materials and methods
94
95 ### 2.1 Plant material and growth conditions
96
97 All experiments were performed on rooted cuttings of hybrid poplar clones (*Populus*
98 *trichocarpa* x *Populus deltoides*) ‘Beaupré’, ‘Boelare’ and the *Populus nigra* clone
99 ‘Vereecken’. Cuttings were obtained from Rothamsted Research Institute, Hertfordshire, UK.

100 The cuttings of about 30cm length were grown in plastic pots containing compost in a
101 greenhouse at the Department of Plant and Soil Sciences, University of Aberdeen. Cuttings
102 for each clone were obtained from a single tree to minimize variations among shoots within a
103 clone. Plants were watered as required and a liquid fertilizer containing most of the important
104 nutrients required for plant growth was applied weekly.
105

106 **2.2 Rust isolates**

107 Rust isolate 16B was obtained from Rothamsted Research Institute, Hertfordshire, UK and
108 maintained at -15$^0$ C. The infection types of the rust isolate were assigned from 0 (immune)
109 to 4 (highly susceptible) according to Pei *et al*. (1996).
110 The clone 'Vereecken' was highly susceptible (infection type 4) to the isolate 16B while the
111 clones 'Beaupré' and 'Boelare' were immune (infection type 0) to the same isolate.
112
113

| 114 | **Poplar clone** | **Rust isolate 16B** |
|---|---|---|
| 115 | *P. deltoids* x *P. trichocarpa* 'Beaupré' | 0 - immune |
| 116 | *P. deltoids* x *P. trichocarpa*, 'Boelare' | 0 - immune |
| 117 | *P. nigra* 'Vereecken' | 4 - susceptible |

118
119

120 **2.3 L-form bacteria and growth conditions**

121 Stable L-form bacteria derived from the cell-walled form of *Bacillus subtilis* NCIMB 8054,
122 ATCC 6633 (Allan, 1991; Allan *et al*, 1993) were maintained on L-phase medium (LPM)
123 (Allan, 1991) supplemented with 5% (v/v) inactivated horse serum (HS) (Gibco, UK). Liquid
124 cultures were initiated by inoculating agar blocks (approx. 2 x 2 cm) containing the good
125 surface growth of L-forms from a 2day old streak plate into L-phase broth (LPB). Cultures on
126 LPM were maintained at 30º C. Liquid cultures were maintained at 30º C in a shaking
127 incubator (Gallenkamp, UK) at the speed of 60 rev min$^{-1}$.
128

129 **2.4 Association of *B. subtilis* L-forms with poplar leaves**

130 The clones 'Vereecken' and 'Beaupré' were used for the association of L-form bacteria under
131 greenhouse conditions. The surface growth of L-form bacteria from a 3d old LPM plates was
132 harvested with 5 % (w/v) sterile mannitol solution. Optical density OD600

133 (Spectrophotometer, CE1010, CECIL, Cambridge, UK) of the L-form suspension was
134 adjusted to approx. 0.7 (approx. $10^7$ CFU $ml^{-1}$). Fully expanded mature leaves in healthy
135 shoots of randomly identified plants (three plants) were selected (3 shoots from each plant).
136 Approximately 20 petioles of selected leaves of the same maturity were treated by injecting
137 200 µl of L-form suspension at a single site closer to the attachment point of the mother plant
138 (2 cm from the attachment point) using a hypodermic syringe and a 23G needle. A similar
139 number of petioles in control plants were treated identically with 5% (w/v) mannitol. Two
140 sets of plants were treated with L-forms and mannitol solution for leaf assays. The petioles of
141 all injected leaves were tagged accordingly. All plants were maintained in the greenhouse at
142 the Department of Plant and Soil Sciences, University of Aberdeen. The leaves were
143 collected for the leaf disc assay from plants after 5 and 10 days of injecting L-forms and
144 mannitol respectively.
145

146 **2.5 Leaf disc assay**

147 The method of Pei *et al*. (2004) was followed with some modifications. Leaves (3-5) were
148 collected from two sets of plants of both clones ('Vereecken' and 'Beaupré' ) after 5 and 10
149 days of injecting L-forms and mannitol solution respectively. Three types of leaves; L-form
150 injected leaves, non-injected leaves in L-form injected plants and mannitol injected leaves
151 were collected. Fifteen leaf discs of 16 mm diameter (5 from each leaf) were punched from
152 number 10 cork borer. The leaf discs were kept (abaxial surface up) on sterile blotting paper
153 bridges soaked in sterile distilled water in 25 (5 x 5) compartments of 10 x 10 $cm^2$ square
154 Petri dishes. To each compartment of the Petri dish, 1.5ml of sterile distilled water (SDW)
155 was added before placing the blotting paper bridge and the leaf disc. The spores of the rust
156 isolate 16B were suspended in SDW containing Tween 20 (1 drop for 100 ml) and the
157 concentration of the spore suspension was adjusted the of 30,000 spores $ml^{-1}$. Each of the 15
158 leaf discs in a single Petri dish was inoculated with 50 µl of the spore suspension (4 droplets
159 on each disc) by means of a sterile micropipette. Petri dishes were incubated in a growth
160 cabinet at $16^0$ C with 16 h $day^{-1}$ illumination. Leaf discs were observed daily for 13 days after
161 rust inoculation for the appearance of uredinia.
162

163 **2.6 Association of *B. subtilis* L-forms with stems of young poplar plants**

164 The clone 'Vereecken' was used for the association of L-form bacteria under greenhouse
165 conditions. The L-form suspension was prepared as previous experiment. The young stems

166 were treated by injecting 200 µl of L-form suspension at a single site using a hypodermic
167 syringe and a 25G needle. A similar number of branches were treated identically with 5%
168 (w/v) mannitol. All injected branches were tagged accordingly. Both treated and control
169 plants were maintained in the same greenhouse. The leaves were collected from both treated
170 and control plants after 10 and 15 days of the treatment. The leaf disc assay was carried out
171 similar to the previous experiment using the spores of *Melampsora* isolate 16B.
172

173 **2.7 Data recorded**

174 The discs were observed daily for the disease development for 13 days from the day of
175 inoculation. Latent period was recorded as the time (days) from inoculation of the rust spores
176 to the first appearance of uredinia on leaf discs. The pustules on each leaf disc were counted
177 (three replicates, each containing 5 leaf discs) daily and the number of pustules per leaf disc
178 was calculated. The number of days until the appearance of the first symptom (incubation
179 period) was also calculated. Thirteen days after inoculation, the distribution of pustules on
180 leaf discs were recorded using a digital camera. Data were analysed using T Test Excel Data
181 Analysis Tool Pak.
182
183

184 **3 Results and Discussion**

185
186 Stable L-forms derived from the cell walled form of *Bacillus subtilis* NCIMB 8054, ATCC
187 6633 have previously been shown to associate with plants such as Strawberry (Ferguson et
188 al., 2000) and Chinese cabbage (Tsomlexoglou *et al.,* 2003). Similar to the work done by
189 Ferguson *et al*., ( 2000) in this study also leaves and stems of 3 month old Poplar plants were
190 injected with L-forms of *B. subtilis* and 5% mannitol as treated and control tests and grown
191 under greenhouse conditions.
192

193 The development of rust symptoms on leaf discs obtained from treated and control plants and
194 inoculated with *Melampsora* spore suspension was monitored by counting the pustule
195 number, by observing the discs under the light microscope (Mazurek, UK). Of the two poplar
196 clones selected, the clone *Populus trichocarpa* x *Populus deltoides* ‘Beaupré’ was immune to
197 the rust isolate 16B (Pei, M.H.,pers. comm.) and this clone showed an incompatible
198 interaction between the pathogen and the host plant. Therefore no visible rust symptoms were

199 appeared on any of the leaf discs of ‘Beaupré’ obtained from the leaves injected with L-form
200 bacteria or mannitol with *Melampsora* isolate 16B. No necrosis was observed, but darker
201 regions were developed and remained restricted to the inoculated areas of leaf discs.
202
203 The *P. nigra* clone ‘Vereecken’ was highly susceptible to the same rust isolate. By 5$^{th}$ day of
204 inoculation with *Melampsora* spores, chlorotic regions were observed on the lower surface of
205 many leaf discs and these were localized to the areas where the droplets of rust spores were
206 kept. On the 7$^{th}$ day, uredinia were readily seen on the leaf discs as yellow to orange pustules
207 (Fig 1).
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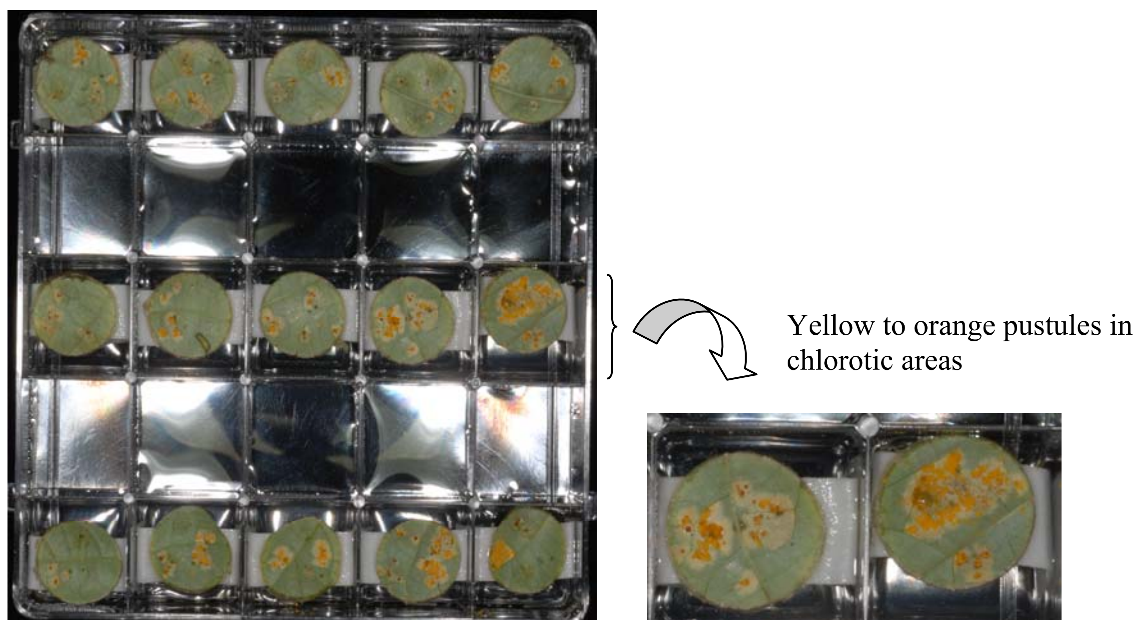


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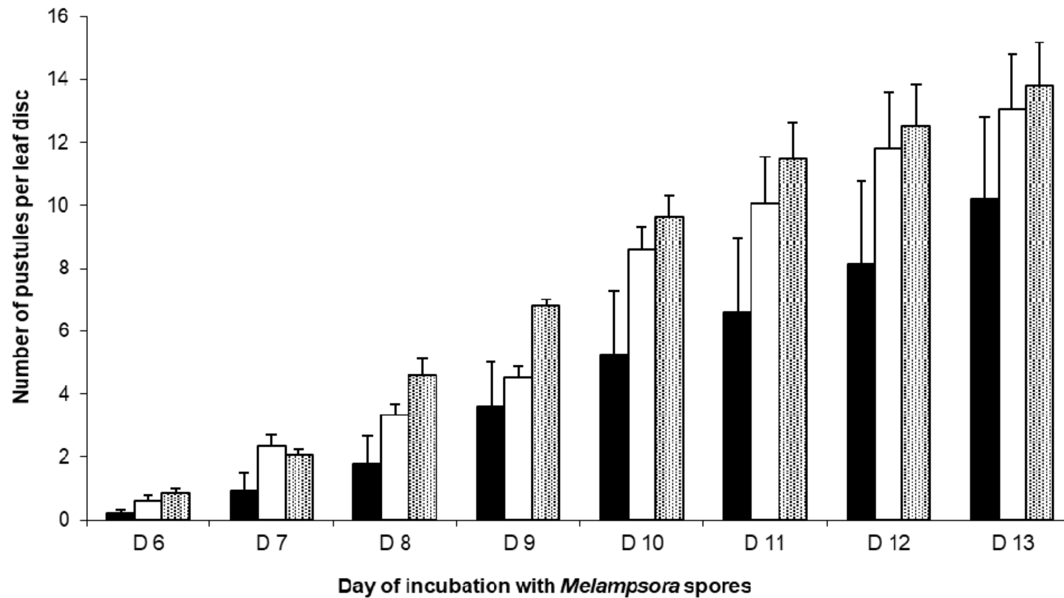
211 **Figure 1** Pale yellow to orange rust pustules (uredinia) developed on the abaxial surface of
212 leaf discs of clone ‘Vereecken’ inoculated with spores of *Melampsora* isolate 16B and
213 incubated in a growth cabinet. Leaf discs were punched from non-injected leaves obtained
214 from plants where another set of selected leaves were injected with L-form bacteria.
215
216 Counting of pustules on leaf discs was started after 5d of inoculation of rust spores as there
217 was no visible disease development on discs during the first five days of inoculation (latent
218 period). The average numbers of pustules developed from D6 to D13 in leaf discs obtained
219 after 5 and 10d of injecting L-form bacteria and mannitol to the leaf petioles are shown in
220 Figures 2 and 3.

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222 Figure 2 - The average number of pustules developed in leaf discs of clone 'Vereecken'
223 during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were
224 prepared from leaves obtained after 5 days of injecting L-form bacteria and mannitol to the
225 leaf petioles ( ■ - L-form injected leaves; □ - Non-injected leaves from L-form injected
226 plants; ▦ - Mannitol injected leaves). Average number of pustules of fifteen leaf discs per
227 each treatment is shown. Error bars represent standard errors of number of pustules of fifteen
228 leaf discs.
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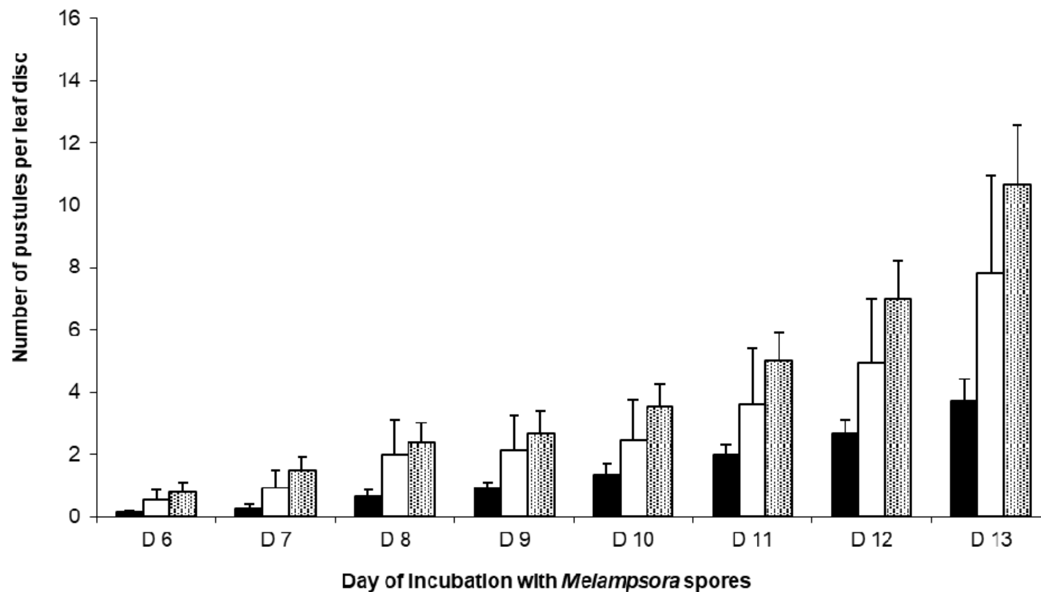


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232 Figure 3 - The average number of pustules developed in leaf discs of clone 'Vereecken'
233 during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were
234 prepared from leaves obtained after 10 days of injecting L-form bacteria and mannitol to the

235
236 leaf petioles ( ■ - L-form injected leaves; □ - Non-injected leaves from L-form injected
237 plants; ▨- Mannitol injected leaves). Average number of pustules of fifteen leaf discs per
238 each treatment is shown. Error bars represent standard errors of number of pustules of fifteen
239 leaf discs.
240
241
242
243 The leaf disc assay showed that the discs obtained from the leaves where the petioles were
244 treated with L-forms had the minimum average number of pustules followed by the
245 non- injected leaves obtained from the same set of plants. Comparing the leaves collected
246 after 5 and 10 days of injecting L-form bacteria (Figures 2 and 3 respectively), pustules
247 developed in all three sets of leaves (L-form injected leaves, non-injected leaves in L-form
248 injected plants and mannitol injected leaves) collected 10 days after injecting L-forms were
249 significantly lower in numbers than that of 5 days. The highest number of pustules was
250 visible on leaf discs obtained from mannitol treated leaves. This indicates that the leaves
251 treated with L-form bacteria were protected from subsequent inoculation of the pathogen
252 *Melampsora.* At the same time L-forms have moved within the plant from the injected leaves
253 to the non –injected leaves showing lower number of pustules in these leaves than the
254 mannitol treated leaves in control plants. The L-forms have greatly reduced rust severity
255 within the inoculated leaves (i.e. local effects), but they had low systemic effect on rust of
256 non-inoculated leaves collected from the L-form inoculated plants. Previously it had been
257 postulated that L-forms of *Pseudomonas syringae* might have evoked a systemic acquired
258 resistance response (Hammerschmidt , 1999) to provide protection against pathogenic
259 bacteria in both Chinese cabbage and bean (Amijee *et al*., 1992; Waterhouse *et al*., 1996).
260 Perhaps different control mechanisms might have occurred depending on the type of bacteria
261 and/or the plant.
262
263 There is previous evidence to prove the movement of L-form bacteria of *B. subtilis* within the
264 plant tissues from the point of their inoculation (Ferguson et al., 2000; Tsomlexoglou et al.,
265 2003). An ELISA that was selective for the L-forms of *Bacillus subtilis* showed that L-forms
266 have moved up to 32-42 cm along the stolon tissues of strawberry form the point of injection.
267 Within 4 days of infection, the L-forms were detected in leaves and new stolons showing
268 their systemic distribution. (Ferguson *et al*., 2000). Daulagala and Allan (2003) reported that
269 when Chinese cabbage seeds were imbibed in suspension of *Pseudomonas syringae* pv.
270 *phaseolicola* NVRS 1281 L-forms and grown in pots and subsequently sprayed with a

271 conidial suspension of *B. cinerea,* leaves of 31 days old seedlings showed consistently lower
272 disease development than the mannitol treated seedlings. However, similar to the work done
273 by Ferguson *et al*. (2000), in this study, the L-forms were injected to the stems and petioles,
274 rather than imbibed during seed germination. During injection, since there is a great
275 possibility for the bacteria to enter the vascular system or the intercellular spaces via the
276 wound and systemically distribute within the plant comparatively within a shorter period of
277 time than the seed imbibition, leaves were collected in this study, after 5, 10 and 15 days of
278 injecting L-forms and mannitol solution.
279
280 Considering the observations of leaf disc assay performed using the leaves obtained from
281 plants where the L-forms and mannitol were injected to stems, significantly lower number of
282 *Melampsora* pustules was observed in leaves from L-form treated plants collected 10 days
283 after the treatment (Fig. 4) than that of 15 days (Fig. 5).
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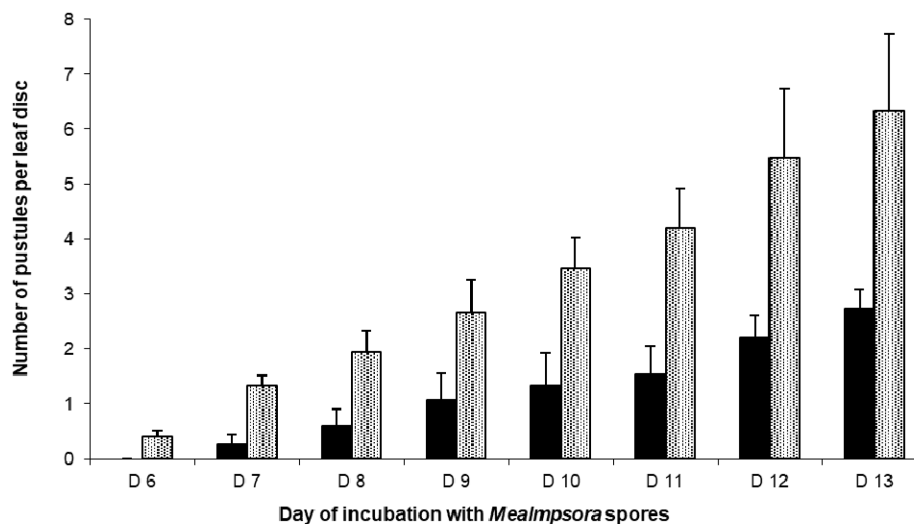


287
288 Figure 4 - The average number of pustules developed in leaf discs of clone
289 'Vereecken' during D6 to D13 incubation with *Melampsora* spores. For inoculation of
290 spores, discs were prepared from leaves obtained after 10 days of injecting L-form bacteria
291 and mannitol to the plant stem ( ■ - leaves obtained from L-form injected plants; ▩ -
292 leaves obtained from Mannitol injected plants). Average number of pustules of fifteen leaf
293 discs per each treatment is shown. Error bars represent standard errors of number of
294 pustules of fifteen leaf discs.
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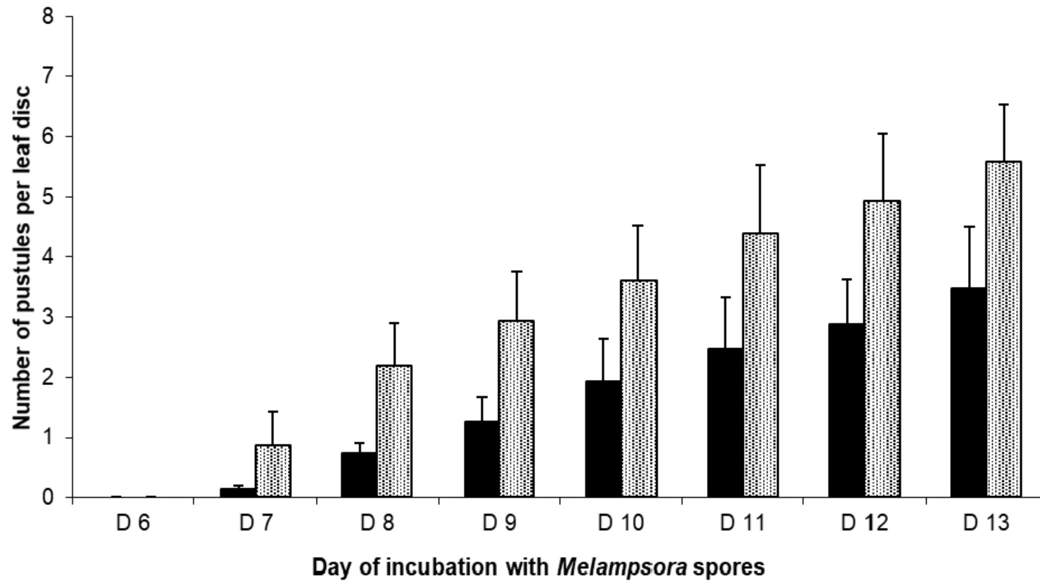


Figure 5 - The average number of pustules developed in leaf discs of clone 'Vereecken' during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were prepared from leaves obtained after 15 days of injecting L-form bacteria and mannitol to the stem ( ■ - leaves obtained from L-form injected plants; ▩ - leaves obtained from Mannitol injected plants). Average number of pustules of fifteen leaf discs per each treatment is shown. Error bars represent standard errors of number of pustules of fifteen leaf discs.

A common reaction of many plants in response to attack by pathogens is the synthesis of pathogenesis- related (PR) proteins. Chitinases as one of the major pathogenesis related proteins have been suggested to play a major role in defence responses of plants against pathogen attack. In combination with β -1,3 glucanases, chitinases lyse hyphal tips of fungi (Daulagala, 2014) or involved in releasing elicitorsthat could activate plant defence mechanisms (Ryan, 1988). When L-forms of *Pseudomonas syringae* pv. *phaseolicola* NVRS 1281 were associated with Chinese cabbage seedlings, compared with the mannitol treated control seedlings, a significant induction of chitinolytic enzymes was detected in seedlings with 4-methylunbelliferyl substrates (Daulagala and Allan, 2003).

297 ## 4. Conclusion

298 This current research clearly revealed that L-forms of *B. subtilis* NCIMB 8054, ATCC 6633
299 could enhance the disease suppression and protect the poplar plants against the leaf rust
300 pathogen *M. larici*-populina. Thus, it could be concluded that L-forms of *B. subtilis* may be
301 useful to control leaf rust disease in poplars as a safe and eco-friendly alternative option to

302 chemical fungicides. According to the previously reported research findings of L-form plant
303 associations, L-forms have protected plants against both bacterial and fungal pathogens in a
304 manner similar to systemic acquired resistance associated with the induction and expression
305 of PR proteins in plants. Therefore, further work is needed to investigate the nature of this L-
306 form-plant symbiosis and the mechanism of protection of poplars against the pathogen *M.*
307 *larici-populina.*

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309 **Consent & Ethical: NA**

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