Short Research Article

Induction of resistance in poplar to *Melampsora larici-populina* using L-form bacteria

Abstract

Poplar (*Populus* spp.) is a deciduous tree that belongs to the Family Salicaceae and grows in most parts of the northern hemisphere from subarctic to subtropical regions. Poplars are susceptible for a variety of bacterial and fungal diseases and the rust caused by *Melampsora* sp. is one of the most serious diseases of poplars. Symbiosis of stable L-form bacteria of *B. subtilis* with rooted poplar cuttings grown under greenhouse conditions inhibited the development of rust pustules on leaves on subsequent exposure to *Melampsora larici-populina*. The development of rust symptoms was monitored by counting the pustule number, by observing the leaf discs under the light microscope. This plant- L-form symbiosis shows an antagonism to fungal pathogen and this has promising implications for the use of this L-form bacteria as a biocontrol agent for poplars against the rust pathogen.

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

1. Introduction

Plants, like humans and other animals exhibit disease symptoms and these diseases are caused by different animate and inanimate agents. Different approaches may be used to prevent, mitigate or control plant diseases (Pal & Gardener, 2006). Conventional disease control is based on application of various chemicals and resistance breeding. The development of highly effective pesticides seemed to offer instant answers to the threat of disease, but the environmental pollution caused by excessive use and misuse of agrochemicals has led to considerable changes in people's attitudes towards their use. Induced disease resistance is an interesting alternative for the plant protection, which is based on the activation of existing resistance mechanisms in the plants and it is effective against a broad spectrum of plant pathogens (Van Loon *et al*., 1998).

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

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

Poplar (*Populus* spp.) occur naturally in most parts of the northern hemisphere from subarctic to subtropical regions (Bean, 1976). Poplar is a deciduous tree that belongs to the Family Salicaceae. There are about 35 species of poplar trees. This tree grows mostly in temperate climates. Poplar trees grow quickly and provide enough shade. Wood can be used for

numerous purposes such as for the production of plywood, musical instruments like guitars, drums and often used in paper industry.

Poplars are susceptible for a variety of bacterial and fungal diseases. Rust caused by *Melampsora* sp. is one of the most serious diseases of poplars. Among the *Melampsora* species, *M. larici-populina* is the most widespread and frequent rust species described in poplar and the principal rust fungus concern in Britain.. The fungus passes through subsequent stages of its life cycle on an alternate host, larch (*Larix* sp.). Poplar rust is easily recognized by the masses of yellow/orange fungal spores that cover the under surface of the leaves. After a few weeks, the leaves blacken, curl up and fall prematurely. Apart from a reduction of growth due to foliage loss, sometimes there is a failure of current year's shoot maturation. Imperfect maturation can lead to a dieback of the shoot, even to the extent that the entire plant may die.

There is no pesticide approved for use against poplar rust in woodlands in the UK. Trials elsewhere in Europe have indicated that economically acceptable but partial control of the disease can be achieved one or two annual applications of a fungicide with curative and persistent properties. However, chemical control of plant diseases is expensive, sometimes physically impracticable and in many cases environmentally undesirable. Use of resistant clones is one of the best disease-control strategies; however, the number of highly resistant clones is limited, making biological control an attractive disease-control alternative. Therefore, there is no doubt that in the future, disease control in plants, presently provided by chemicals, mainly by fungicides and bactericides, will be replaced by new disease control technologies emerging from the knowledge of plant-microbe interactions.

2. Materials and methods

2.1 Plant material and growth conditions

All experiments were performed on rooted cuttings of hybrid poplar clones (*Populus trichocarpa* x *Populus deltoides*) 'Beaupré', 'Boelare' and the *Populus nigra* clone 'Vereecken'. Cuttings were obtained from Rothamsted Research Institute, Hertfordshire, UK and grown in pots containing compost in a greenhouse at the Department of Plant & Soil Sciences, University of Aberdeen.

2..2 Rust isolates

Rust isolate 16B was obtained from Rothamsted Research Institute, Hertfordshire, UK and maintained at -15^0 C. The infection types of the rust isolate were assigned from 0 (immune) to 4 (highly susceptible) according to Pei *et al*. (1996).

The clone'Vereecken' was highly susceptible (infection type 4) to the isolate 16B while the clones 'Beaupré' and 'Boelare' were immune (infection type 0) to the same isolate.

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

2.3 L-form bacteria and growth conditions

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

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

The clones 'Vereecken' and 'Beaupré' were used for the association of L-form bacteria under greenhouse conditions. The surface growth of L-form bacteria from a 3d old LPM plates was harvested with 5 % (w/v) sterile mannitol solution. Optical density OD600 (Spectrophotometer, CE1010, CECIL, Cambridge, UK) of the L-form suspension was adjusted to approx. 0.7 (approx. 10^7 CFU ml^{-1}). Fully expanded mature leaves from healthy shoots were selected (3 shoots from each plant). Approximately 20 petioles of selected leaves at same maturity on three shoots were treated by injecting 200 µl of L-form suspension at a single site closer to the attachment point to the mother plant (2 cm from the attachment point) using a hypodermic syringe and a 23G needle. A similar number of petioles in control plants were treated identically with 5% (w/v) mannitol. Two sets of leaves were treated with L-

forms and mannitol solution for leaf assays. The petioles of all injected leaves were tagged accordingly. All plants were maintained in the greenhouse at the Department of Plant & Soil Sciences, University of Aberdeen. The leaves were collected for the leaf disc assay from plants after 5 and 10 days of injecting L-forms and mannitol respectively.

2.5 Leaf disc assay on leaves injected with L-form bacteria and mannitol

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

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

The clone 'Vereecken' was used for the association of L-form bacteria under greenhouse conditions. The L-form suspension was prepared as previous experiment. The young stems were treated by injecting 200 µl of L-form suspension at a single site using a hypodermic syringe and a 25G needle. A similar number of branches were treated identically with 5% (w/v) mannitol. All injected branches were tagged accordingly. Both treated and control plants were maintained in the same greenhouse. The leaves were collected from both treated and control plants after 10 and 15 days of the treatment. The leaf disc assay was carried out similar to the previous experiment using the spores of *Melampsora* isolate 16B.

2.7 Disease assessment

The discs were observed daily for the disease development for 13 days from inoculation. The pustules on each leaf disc were counted (three replicates) daily and the number of pustules per leaf disc was calculated. The number of days until the appearance of the first symptom (incubation period) was also calculated. Thirteen days after inoculation, the distribution of pustules on leaf discs were recorded using a digital camera.

3 Results and discussion

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

The development of rust symptoms on leaf discs obtained from treated and control plants and inoculated with *Melampsora* spore suspension was monitored by counting the pustule number, by observing the discs under the light microscope (Mazurek, UK). Of the two poplar clones selected, the clone *Populus trichocarpa* x *Populus deltoides* 'Beaupré' was immune to the rust isolate 16B (Pei, M.H.,pers. comm.) and this clone showed an incompatible interaction between the pathogen and the host plant. Therefore no visible rust symptoms were appeared on any of the leaf discs of 'Beaupré' obtained from the leaves injected with L-form bacteria or mannitol with *Melampsora* isolate 16B. No necrosis was observed, but darker regions were developed and remained restricted to the inoculated areas of leaf discs.

The *P. nigra* clone 'Vereecken' was highly susceptible to the same rust isolate and showed a compatible interaction. By 5 days after inoculation with *Melampsora* spores, chlorotic regions were observed on the lower surface of many leaf discs and these were localized to the areas where the droplets of rust spores were kept. By 7 days, uredinia were readily seen on the leaf discs as orange pustules (Fig 1).

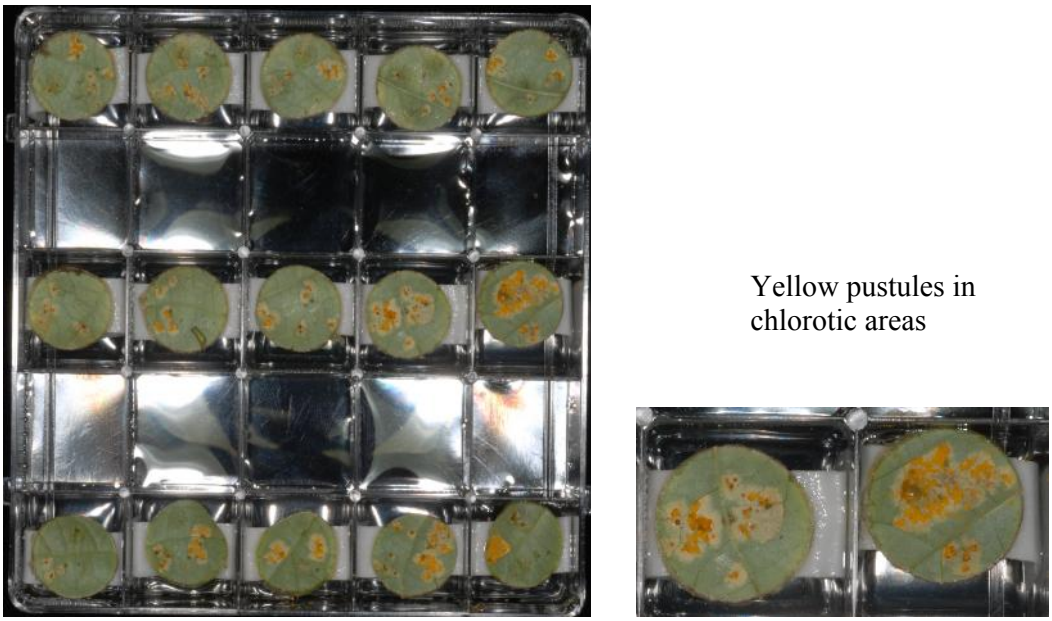

Figure 1 Development of chlorotic areas and rust pustules on leaf discs of clone 'Vereecken' (obtained from leaves where petioles were injected with L-form bacteria) inoculated with spores of *Melampsora* isolate 16B.

Counting of pustules on leaf discs was started after 5d of inoculation of rust spores as there were no visible disease development on discs during the first five days of inoculation. The average numbers of pustules developed from D6 to D13 in leaves after 5 and 10d of injecting L-form bacteria and mannitol to the leaf petioles are shown in Figures 2 and 3.

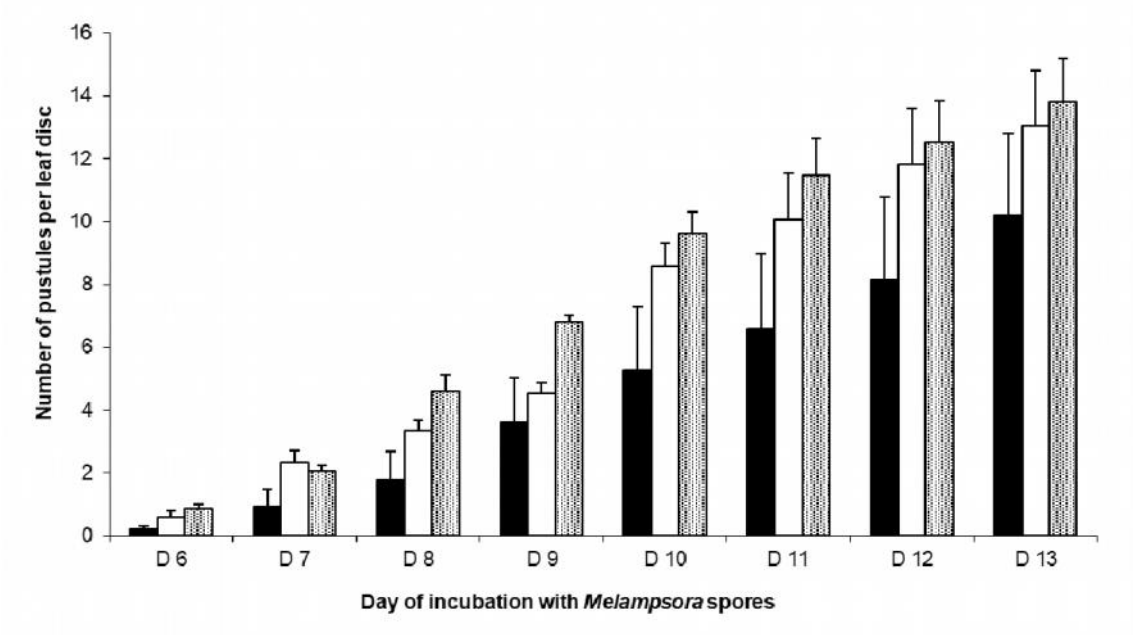

Figure 2 - The average numbers of pustules developed from D6 to D13 in leaves of clone 'Vereecken' after 5 days of injecting L-form bacteria and mannitol to the leaf petioles (■ - L-form injected leaves; □ - Non-injected leaves from L-form injected plants; ▨ - Mannitol injected leaves). Average number of pustules of fifteen leaf discs per each treatment is shown. Error bars represent standard errors of number of pustules of fifteen leaf discs.

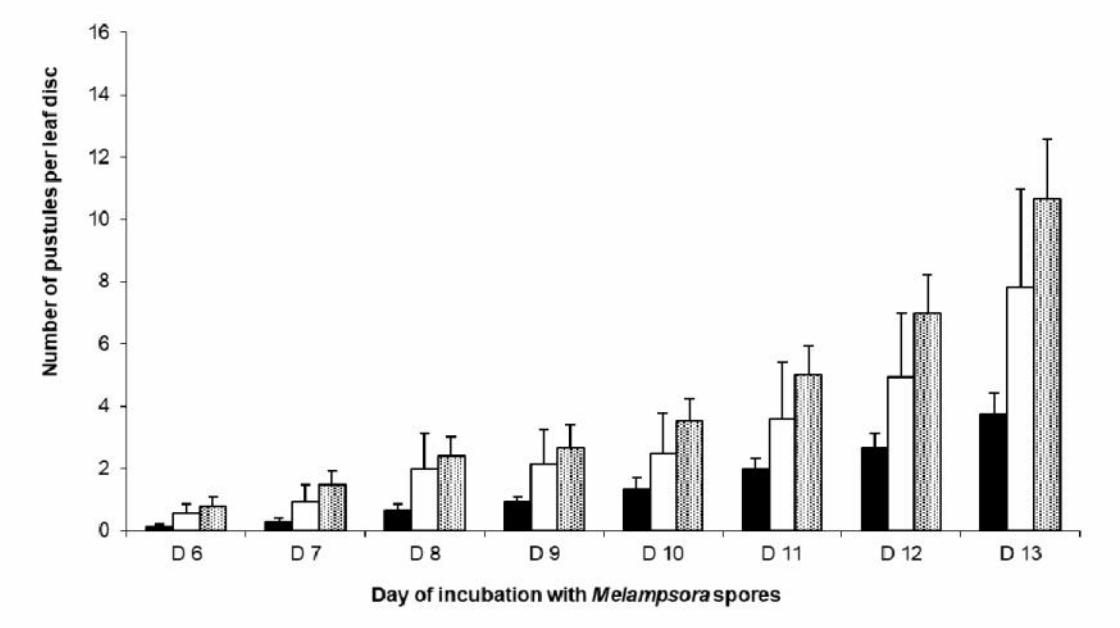

Figure 3 - The average numbers of pustules developed from D6 to D13 in leaves of clone 'Vereecken' after 10 days of injecting L-form bacteria and mannitol to the leaf petioles (■ - L-form injected leaves; □ - Non-injected leaves from L-form injected plants; ▨ - Mannitol injected leaves). Average number of pustules of fifteen leaf discs per each treatment is shown. Error bars represent standard errors of number of pustules of fifteen leaf discs.

The leaf disc assay showed that the discs obtained from the leaves where the petioles were treated with L-forms had the minimum average number of pustules followed by the non- injected leaves obtained from the same set of plants. Comparing the leaves collected after 5 and 10 days of injecting L-form bacteria (Figures 2 and 3 respectively), pustules developed in all three sets of leaves (L-form injected leaves, non-injected leaves in L-form injected plants and mannitol injected leaves) collected 10 days after injecting L-forms were significantly lower in numbers than that of 5 days. The most number of pustules were visible on leaf discs obtained from mannitol treated leaves. This indicates that the leaves treated with L-form bacteria have been shown to be protected from subsequent inoculation of the pathogen *Melampsora* and at the same time L-forms have moved within the plant from the injected leaves to the non –injected leaves showing lower number of pustules in these

236 leaves than the mannitol treated leaves in control plants. Previously it has been postulated
237 that L-forms of Pseudomonas syringae may have evoked a systemic acquired resistance
238 response (Hammerschmidt, 1999) to provide protection against pathogenic bacteria in both
239 Chinese cabbage and bean (Amijee *et al*., 1992; Waterhouse *et al*., 1996) but perhaps
240 different control mechanisms may occur depending on the type of bacteria and/or the plant.
241
242 There is previous evidence to prove the movement of L-form bacteria of *B. subtilis* within the
243 plant tissues from the point of their inoculation (Ferguson et al., 2000; Tsomlexoglou et al.,
244 2003). An ELISA that was selective for the L-forms of *Bacillus subtilis* showed that L-forms
245 have moved up to 32-42 cm along the stolon tissues of strawberry form the point of injection.
246 Within 4 days of infection, the L-forms were detected in leaves and new stolons showing
247 their systemic distribution. (Ferguson et al., 2000). Daulagala & Allan (2003) reported that
248 when Chinese cabbage seeds were imbibed in suspension of *Pseudomonas syringae* pv.
249 *phaseolicola* NVRS 1281 L-forms and grown in pots and subsequently sprayed with a
250 conidial suspension of *B. cinerea,* leaves of 31 days old seedlings showed consistently lower
251 disease development than the mannitol treated seedlings. However, similar to the work done
252 by Ferguson *et al*. (2000), in this present study, the L-forms were injected to the stems and
253 petioles, rather than imbibed during seed germination. During injection, since there is a great
254 possibility for the bacteria to enter the vascular system or the intercellular spaces via the
255 wound and systemically distribute within the plant, in this present study, leaves collected
256 after 5, 10 and 15 days of injecting L-forms and mannitol solution.
257
258 Considering the observations of leaf disc assay performed using the leaves obtained from
259 plants where the L-forms and mannitol were injected to stems, significantly lower number of
260 *Melampsora* pustules were developed on leaves from L-form treated plants collected 10 days
261 after the treatment (Fig. 4) than that of 15 days (Fig. 5).

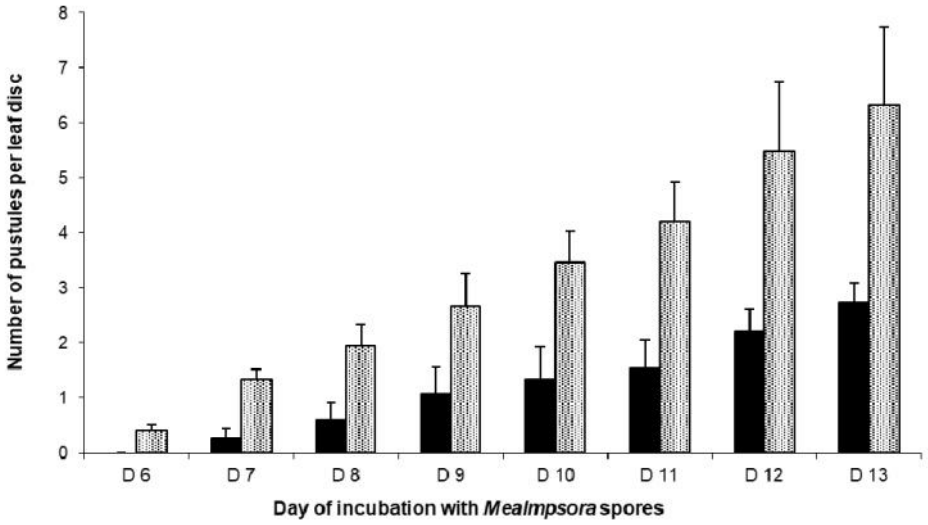

Figure 4 - The average numbers of pustules developed from D6 to D13 in leaves of clone 'Vereecken' after 10 days of injecting L-form bacteria and mannitol to the stem (■ - leaves obtained from L-form injected plants; ▨ - leaves obtained from Mannitol injected plants). Average number of pustules of fifteen leaf discs per each treatment is shown. Error bars represent standard errors of number of pustules of fifteen leaf discs.

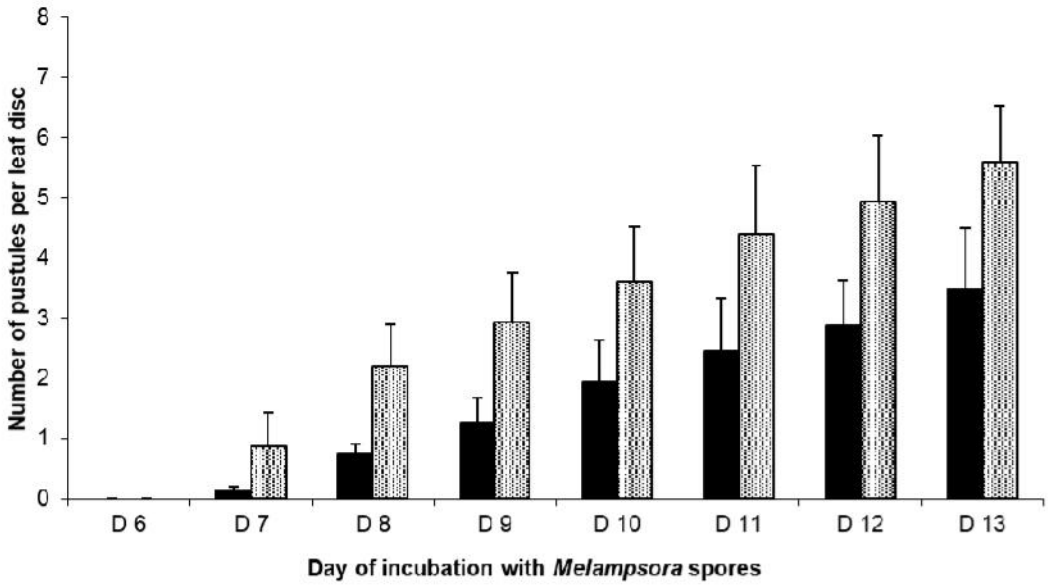

Figure 5 - The average numbers of pustules developed from D6 to D13 in leaves of clone 'Vereecken' after 15 days of injecting L-form bacteria and mannitol to the stem (■ - leaves obtained from L-form injected plants; ▨ - leaves obtained from Mannitol injected plants). Average number of pustules of fifteen 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 elicitors that can 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-methylunbelliferal substrates (Daulagala & Allan, 2003).

4. Conclusion

This current research clearly revealed that L-form bacteria can protect poplar plants against the pathogen *Melampsora larici*-populina which causes rust disease in poplars. This finding suggests that L-form bacteria offer an alternative biocontrol strategy for pathogenic microorganisms. According to the previously reported research findings of L-form plant associations, L-forms have protected plants against both bacterial and fungal pathogens in a

manner similar to systemic acquired resistance associated with the induction and expression of PR proteins in plants. Therefore, further work is needed to investigate the nature of this L-form-plant symbiosis and the mechanism of protection of poplar plants against the fungal pathogen *Melampsora larici*-populina.

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