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Induction of Resistance in Poplar to *Melampsora larici-populina* Using L-form Bacteria

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

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

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Keywords: 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, become diseased and these diseases are caused by different animate and inanimate agents. Different approaches are used to prevent, mitigate or control plant diseases [1]. Conventional disease control is based on the application of various chemicals and resistance breeding. The development of highly effective pesticides seem to offer instant solutions to the threat of disease, but the environmental pollution caused by excessive use and misuse of agrochemicals has changed 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 [2].

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 [2,3]. Expression of localized necrosis caused by the inducing pathogen is the major characteristic of SAR. This necrosis can be either a hypersensitive 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) [3,4]. ISR is induced by certain strains of plant growth promoting rhizobacteria (PGPR). Unlike SAR, ISR is not associated with necrosis. ISR depends on the perception of ethylene and jasmonic acid and it is not associated with expression of genes for PR proteins [2].

L-form bacteria have modified or no cell walls [5] and are capable of forming non-pathogenic symbioses with a wide range of plants [6]. This type of symbiosis confers resistance against subsequent challenge by other fungal and bacterial pathogens [7,8] and hence the association has the potential as a novel system for biological control. However, the mechanisms for the protection are not well known. But Daulagala and Allan [8] detected a higher activity of chitinase, a major PR protein of L-form treated Chinese cabbage plants than that of the control plants treated with 5% (w/v) mannitol and this suggested that L-forms have induced the activity of chitinases 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. Furthermore, the resistance expressed by the L-forms was similar to the resistance observed in plants colonized with the mutants of *Colletotrichum magna*, which were no longer pathogenic, but very successfully colonized a wide range of host plants as endophytes [9]. 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 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. *M. larici-populina* is an obligate macrocyclic basidiomycete, which has its sexual stage on larch (*Larix* sp.) and its asexual stage on poplar. Poplar rust is easily recognized by the masses of yellow/orange fungal spores that cover the under the 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 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 by 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. This present study was conducted to determine if *M. larici-populina* in poplars could be controlled using L-form bacteria of *B. subtilis* as a safe and alternative strategy to reduce the dependency on synthetic fungicides.

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

2.2 Rust Isolates

Rust isolate 16B was obtained from Rothamsted Research Institute, Hertfordshire, UK and maintained at -15°C. The infection types of the rust isolate were assigned from 0 (immune) to 4 (highly susceptible) according to [10].

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 [11,12] were maintained on L-phase medium (LPM) [11] 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 the good surface growth of L-forms from a 2day old streak plate into L-phase broth (LPB). Cultures on LPM were maintained at 30°C. Liquid cultures were maintained at 30°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 in healthy shoots of randomly identified plants (three plants) were selected (3 shoots from each plant). Approximately 20 petioles of selected leaves of the same maturity were treated by injecting 200 µl of L-form suspension at a single site closer to the attachment point of 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 plants 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 and 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

The method of [13] was followed with some modifications. Leaves (3-5) were collected from two sets of plants of both clones ('Vereecken' and 'Beaupré') after 5 and 10 days of injecting L-forms and mannitol solution respectively. Three types 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 leaf) were punched from 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, 1.5 ml 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 as of 30,000 spores ml^{-1}. Each of the 15 leaf discs in a single Petri dish was 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°C with 16 h day^{-1} illumination. Leaf discs were observed daily for 13 days after rust inoculation for the appearance of uredinia.

2.6 Association of *B. subtilis* L-forms with Stems of Young Poplar Plants

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 Data Recorded

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

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 [14] and Chinese cabbage [15]. Similar to the work done by [14] in this study also leaves and stems of 3 month 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. By 5^{th} day of 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. On the 7^{th} day, uredinia were readily seen on the leaf discs as yellow to orange pustules (Fig. 1).

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

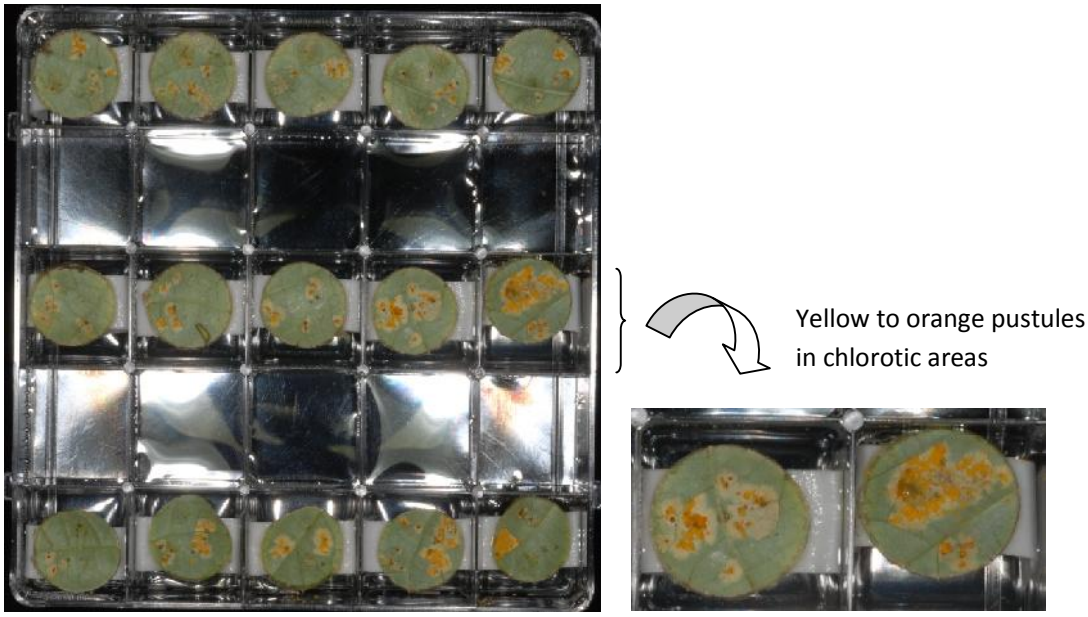

Fig. 1. Pale yellow to orange rust pustules (uredinia) developed on the abaxial surface of leaf discs of clone 'Vereecken' inoculated with spores of *Melampsora* isolate 16B and incubated in a growth cabinet. Leaf discs were punched from non-injected leaves obtained from plants where another set of selected leaves were injected with L-form bacteria

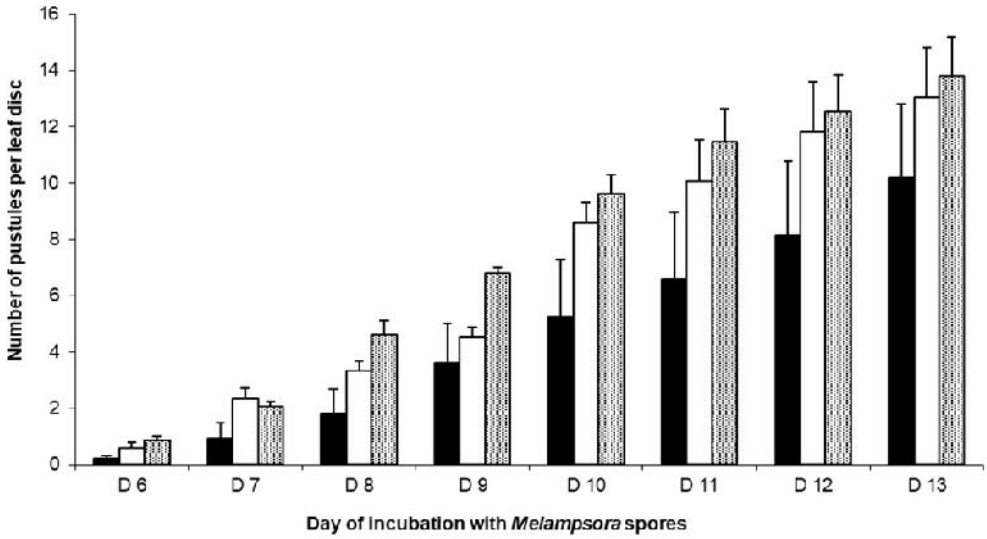

Fig. 2 . The average number of pustules developed in leaf discs of clone 'Vereecken' during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were prepared from leaves obtained after 5 days of injecting L-form bacteria and mannitol to the leaf petioles (■- L-form injected 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

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 (Figs. 2 and 3 respectively), pustules developed in all three sets

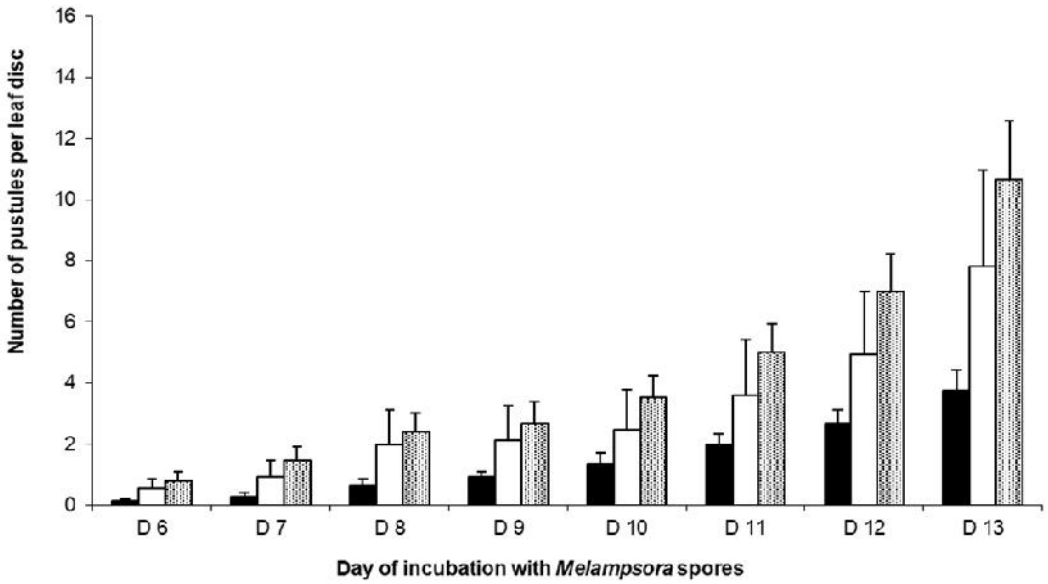

Fig. 3. The average number of pustules developed in leaf discs of clone 'Vereecken' during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were prepared from leaves obtained after 10 days of injecting L-form bacteria and mannitol to the leaf petioles (■ - L-form injected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

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 highest number of pustules was visible on leaf discs obtained from mannitol treated leaves. This indicates that the leaves treated with L-form bacteria were protected from subsequent inoculation of the pathogen *Melampsora.* 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 leaves than the mannitol treated leaves in control plants. The L-forms have greatly reduced rust severity within the inoculated leaves (i.e. local effects), but they had low systemic effect on rust of non-inoculated leaves collected from the L-form inoculated plants. Previously it had been postulated that L-forms of *Pseudomonas syringae* might have evoked a systemic acquired resistance response [4] to provide protection against pathogenic bacteria in both Chinese cabbage and bean [16,17]. Perhaps different control mechanisms might have occurred depending on the type of bacteria and/or the plant.

There is previous evidence to prove the movement of L-form bacteria of *B. subtilis* within the plant tissues from the point of their inoculation [14,15] . An ELISA that was selective for the L-forms of *Bacillus subtilis* showed that L-forms have moved up to 32-42 cm along the stolon tissues of strawberry form the point of injection. Within 4 days of infection, the L-forms were detected in leaves and new stolons showing their systemic distribution [14]. Further [8] reported that when Chinese cabbage seeds were imbibed in suspension of *Pseudomonas syringae* pv. *phaseolicola* NVRS 1281 L-forms and grown in pots and subsequently sprayed with a conidial suspension of *B. cinerea,* leaves of 31 days old seedlings showed consistently lower disease development than the mannitol treated seedlings. However, similar to the work done by [14], in this study, the L-forms were injected to the stems and petioles, rather than imbibed during seed germination. During injection, since there is a great possibility for the bacteria to enter the vascular system or the intercellular spaces via the wound and systemically distribute within the plant comparatively within a shorter period of time than the seed imbibition, leaves were collected in this study, after 5, 10 and 15 days of injecting L-forms and mannitol solution.

Considering the observations of leaf disc assay performed using the leaves obtained from plants where the L-forms and mannitol were injected to

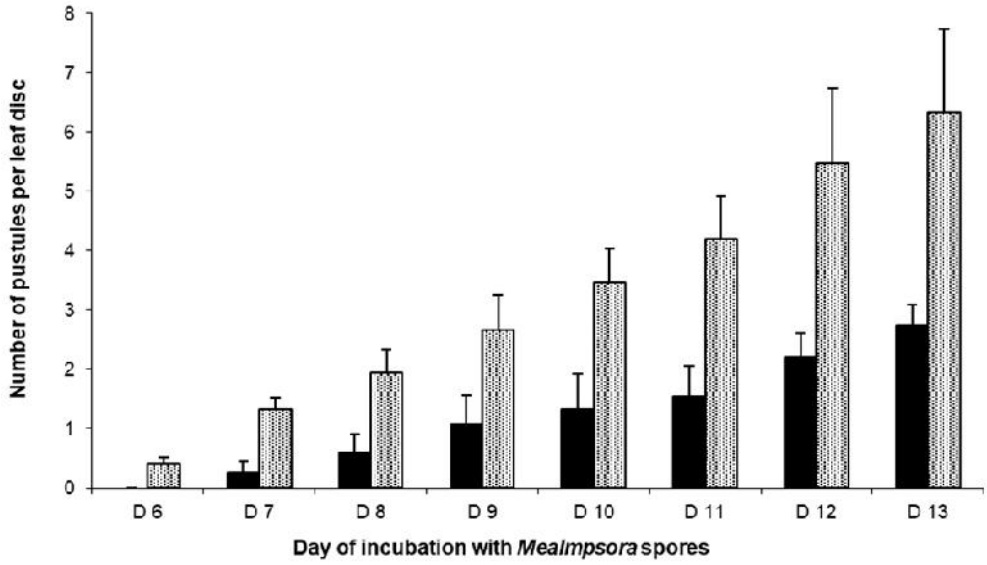

Fig. 4. The average number of pustules developed in leaf discs of clone 'Vereecken' during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were prepared from leaves obtained after 10 days of injecting L-form bacteria and mannitol to the plant stem (■ - leaves obtained 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

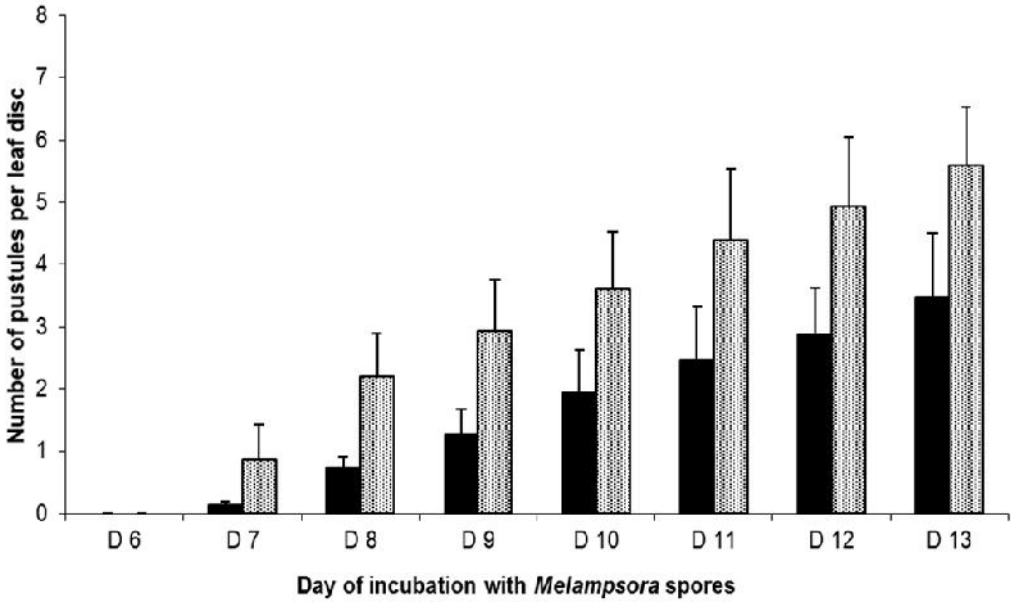

Fig. 5. The average number of pustules developed in leaf discs of clone 'Vereecken' during D6 to D13 incubation with *Melampsora* spores. For inoculation of spores, discs were prepared from leaves obtained after 15 days of injecting L-form bacteria and mannitol to the stem (■- leaves obtained from L-form 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

stems, significantly lower number of *Melampsora* pustules was observed in leaves from L-form treated plants collected 10 days after the treatment (Fig. 4) than that of 15 days (Fig. 5).

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 [18] or involved in elicitors that could activate plant defence mechanisms [19]. 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-methylumbelliferyl substrates [8].

4. CONCLUSION

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

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Pal KK, Gardener BM. Biological control of plant pathogens. The Plant Health Instructor; 2006.
   DOI: 10.1094/PHI-A-2006-1117-02
2. Van Loon LC, Backer PAHM, Pieterse CMJ. Systemic resistance induced by rhizophere bacteria. Annual Review of Phytopathology. 1998;36:433-438.
3. Sticher L, Mauch Mani B, Metraux J P. Systemic acquired resistance. Annual Review of Phytopathology. 1997;35:235-270.
4. Hammerschmidt R. Induced disease resistance: How do induced plants stop pathogens? Physiological and Molecular Plant Pathology. 1999;58:53-53.
5. Madoff S. Introduction to the bacterial L-forms. In: The Bacterial L-forms. New York, Maecel Dekker. 1986;1-20.
6. Paton AM. L-forms: Evolution or revolution? Journal of Applied Bacteriology. 1987;63:365-371.
7. Walker R, Ferguson CMJ, Booth NA. Allan EJ. The symbiosis with *Bacillus subtilis* L-forms with Chinese cabbage seedlings and inhibit conidial germination of *Botrytis cinerea*. Letters in Applied Microbiology. 2002;34:42-45.
8. Daulagala PWHKP, Allan EJ. L-form bacteria of *Pseudomonas syringae* pv. *phaseolicola* induce chitinases and enhance resistance to *Botrytis cinerea* infection in Chinese cabbage. Physiological and Molecular Plant Pathology. 2003;62:253-263.
9. Redman RS, Ranson JC, Rodriguez RJ. Conversion of the pathogenic fungus *Colletotrichum magna* to a non-pathogenic endophytic mutualist by gene disruption. Molecular Plant Microbe Interactions. 1999;12:969-975.
10. Pei MH, Royle DJ, Hunter T. Pathogenic specialisation of *Melampsora epitea var. epitea on Salix.* Plant Pathology. 1996;45: 679–90.
11. Allan EJ. Induction and cultivation of a stable L-form of *Bacillus subtilis*. Journal of Applied Bacteriology.1991;70:339-343.
12. Allan EJ, Amijee F, Tyson RH, Strang JA, Innes CMJ, Paton AM. Growth and physiological characteristics of *Bacillus subtilis* L-forms. Journal of Applied Bacteriology. 1993;74:588-594.
13. Pei MH, Ruiz C, Bayon C, Hunter T. Rust resistance in Salix to *Melampsora larici-epitea*. Plant Pathology. 2004;53:770–779.
14. Ferguson CMJ, Booth NA, Allan EJ. An ELISA for the detection of *Bacillus subtilis* L-form bacteria confirms their symbiosis in strawberry. Letters in Applied Microbiology. 2000;31:390-394.
15. Tsomlexoglou E, Daulagala PWHKP, Gooday GW, Glover LA, Seddon B, Allan EJ. Molecular detection and β-glucuronidase expression of gus-marked *Bacillus subtilis* L-form bacteria in developing Chinese cabbage seedlings.

Journal of Applied Microbiology. 2003;95: 218–224.
16. Amijee F, Allan EJ, Waterhouse RN, Glover LA, Paton AM. Non-pathogenic association of L-form bacteria (*Pseudomonas syringae* pv. *phaseolicola*) with bean plants (*Phaseolus vulgaris* L.) and its potential for biocontrol of halo blight disease. Biocontrol, Science and Technology. 1992;2:203-214.
17. Waterhouse RN, Buhariwalla H, Bourn D, Rattray EJ, Glover LA. CCD detection of *lux* marked *Pseudomonas syringae* pv. *phaseolicola* L-forms associated with Chinese cabbage and the resulting disease protection against *Xanthomonas campestris*. Letters in Applied Microbiology. 1996;22:262-266.
18. Daulagala PWHKP. Expression of chitinase with antifungal activities in ripening Banana fruit. Tropical Plant Research. 2014;1(3):72-79.
19. Ryan CA. Oligosaccharides as recognition signals for the expression of defense genes in plants. Biochemistry. 1988;27: 8879-8883.

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