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Premature germination of resting spores as a means of protecting brassica crops from *Plasmodiphora brassicae* Wor., (Clubroot)

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Running title: Triggering premature spore germination in *Plasmodiophora brassicae*

Abstract

Clubroot disease causes substantial yield and quality losses in broadacre oil seed and intensive vegetable brassica crops worldwide. The causal microbe *Plasmodiophora brassicae* Wor., perennates as soil-borne dormant resting spores. Their germination is triggered by exudates from host roots. A valuable addition to sustainable integrated control strategies could be developed by identifying and synthesising the molecules responsible for stimulating resting spore germination. This paper reports experiments in which stimulatory exudates were collected from brassica roots following exposure to infective stages of *P. brassicae*. Analyses identified a germination signalling molecule of *circa* 1 kDa formed of glucose sub-units. Mass spectral analyses showed this to be a complex hexasaccharide carbohydrate with structural similarities to the components of plant cell walls. This is the first report of a host generated hexasaccharides which is capable of stimulating the germination of resting spores of *P. brassicae*. The implications for environmentally benign control of clubroot are discussed briefly.

Keywords: *Plasmodiphora brassicae,* clubroot, resting spores, germination, hexasaccharide, integrated control

Clubroot disease (*Plasmodiophora brassicae*) causes economically very serious damage to brassica crops worldwide (Dixon, 2009, Strelkov & Dixon, 2014). The disease cycle begins with the germination of environmentally resistant, soil borne resting spores (Dixon 2014). This releases biflagellate naked primary zoospores which swim in soil moisture films towards host root hairs (Aist & Williams, 1971). Once inside a host the pathogen reproduces causing disruption of the host metabolism and the development of swollen root tissues. Severely malformed roots lose their normal functions resulting in premature host death. Eventually the roots decay releasing further generations of resting spores into the soil (Dixon. 2006). This pathogen is most vulnerable to control strategies during the period from resting spore germination to penetration into host root hairs.

The development of crop protection molecules whose mode of action operates by encouraging resting spore germination offers an effective and sustainable means of control. Root exudates were identified as capable of stimulating resting spore germination by Macfarlane, (1970). Subsequently, Craig (1989) showed that root exudates from green broccoli (*B. oleracea* var. *italica*) stimulated resting spore germination. Further research demonstrated that the highest levels of resting spore germination (75%) followed treatment with root exudates derived from susceptible cabbages (*B. oleracea* var. *capitata*) (Ohi *et al,* 2003; Hata *et al*, 2002). The research reported here describes the isolation and identification of the chemical nature of specific germination stimulators derived from root exudates.

Materials and Methods

Heavily galled cabbage (*B. oleracea* var. *capitata*) roots were preserved at -20 ^{o}C until required (Dixon, 1976). Resting spore extraction involved defrosting the roots, washing-off residual soil and homogenising portions in 100 ml aliquots of distilled water. The resultant slurries were filtered through four layers of surgical gauze and 44ml of filtrate containing resting spores was decanted as 1.5ml aliquots into Eppendorf tubes. These were centrifuged at 2000 g for 20 minutes. The resultant spore pellets were clarified by repeated suspension in aliquots of 1ml of distilled water and re-centrifuged. Thereafter, the spore pellets were re-suspended were in distilled water and stored at -20 ^{o}C.

The brassica host used in this research was the clubroot susceptible cabbage (*Brassica oleracea* var. *capitata*) cv. Bartolo seed was obtained from a commercial source. When required seed was germinated in Petri plates lined with surgical gauze each moistened with 5ml distilled water and held in darkness at 20 ^{o}C for two days and then exposed to light. A sample of seedlings was used to determine that the spores of *P. brassicae* obtained by centrifugation and clarification were capable of germination. These were transferred to fresh Petri plates and the roots were sprayed with an aliquot of *P. brassicae* resting spores at a concentration of 10^{7} spores /ml as determine by haemocytometry. After 24 h root samples of 1 cm length were dissected and placed on a microscope slide mounted in fresh distilled water. This was viewed by microscopy and showed that the resting spores had germinated and released primary biflagellate zoospores which were actively swimming around the root samples. This demonstrated that exudates from the roots of cv Bartolo were capable of stimulating the germination of resting spores of *P. brassicae*.

Establishing the chemical nature of the triggers of *P. brassicae* resting spore germination coming from cv Bartolo roots required increased volumes of exudates. Seed was sown into a series of ten Petri plates which were prepared as described above. The resultant germinated plants were allowed to grow in the plates placed in light on a north facing laboratory window ledge for 10 days and water in the plates was replenished as required. At the end of this time the seedlings were carefully removed from the surgical gauze and the water squeezed out into a beaker using forceps. Residual moisture was present on the gauze was expressed by placing it in centrifuge tubes and spinning at 2000g for 10 minutes. In total this yielded 42 ml of fluid which contained root exudates produced by the germinating and growing cv Bartolo seedlings. The fluid had a slightly milky appearance it was stored at 4 °C in a laboratory refrigerator. It was necessary to demonstrate that this fluid contained root exudates capable of stimulating resting spore germination. Approximately 0.5 ml of fluid was pipetted onto each of five microscope slides and an aliquot of the resting spore suspension was added. Each slide was sealed with nail varnish thereby preventing desiccation. After 20 h examination by microscopy identified motile biflagellate primary zoospores of *P. brassicae* actively swimming on the slide. This confirmed the presence of an active compound capable of triggering resting spore germination.

Initial experiments with reverse phase high pressure liquid chromatography (HPLC) and elution with several gradients did not find eluates with germination stimulating properties. Subsequent experiments with a gravity fed gel filtration column (Sephadex 100; 120 cm x 1cm eluted with 0.1M phosphate buffer) recovered a compound which did stimulate resting spore germination. When this stimulant was injected into the column several peaks were obtained in the mass detector trace. The separation was repeated several times using a fraction collector. Fractions of root exudates were collected every 4 minutes. Ultimately 52 tubes were collected each filled with 1 ml eluate. The potency of these fractions for stimulating the germination of resting spores of *P. brassicae* was tested. An aliquot of 0.25ml was taken from each fraction and placed on a microscope slide with a similar volume of *P. brassicae* resting spores suspended in distilled water. The slide was sealed with nail varnish and held at room temperature for 20hr. Microscopic examination identified the presence of swimming biflagellate zoospores of *P. brassicae*. The most active fraction, number 24, stimulated germination in this assay down to 10-fold dilution of the eluates from the fraction collector. This positive fraction had a retention time of 85-89 minutes and a molecular weight of 1 KDa. Bradford's reagent tests indicated the absence of proteins. But the large molecular weight suggested that it was a carbohydrate. The active fraction was frozen and freeze-dried to a white residue for mass spectral assays.

Subsequently, more accurate estimates of molecular weight were obtained by HPLC using a Dionex CarboPac MA-1 analytical column (4 x 250mm) with the guard column Dionex CarboPac MA-1 (4 x 50mm) and Mass Detector Sedex model 55. The set-up details were:- *iso*cratic gradient A:B (%) 15:85; A contained de-ionised water and B contained 600mM sodium hydroxide, flow rate: 0.4ml/min, temperature was ambient, injection: 20μL, run time was 45 minutes and detection used a Dionex ED40 Electrochemical Detector and mass detector Sedex model 55. The system was calibrated with dextrans of differing molecular weights viz: 2,000,000, 298,000, 9,100 and 8,800 Da in 1 mgml^{-1} solutions.

Samples from the gel column were hydrolysed with 2M hydrochloric acid 1:1 v/v at 100° C for 1 hour. Calibration of the column used standard sugar samples, their retention times were: mannose, 20.53; glucose, 22.65 and galactose, 24.97 respectively. The trace from a 1 hr hydrolysis produced a peak in the glucose position. When hydrolysis was extended to 3 hrs the peak reached a maximum. Three repetitions of this analysis each produced a single peak in the glucose position. Mass spectral analysis was made on a Liquid Chromatography Quadrupole (LCQ) Classic machine (Thermoscientific, Hemel Hempstead, Hertfordshire) using an electrospray ionization needle voltage of 4.5kV and capillary temperature of 250 ^{o}C. The sample was introduced in 0.1% v/v aqueous formic acid and the instrument was scanned between 100 and 2,000 atomic mass units (amu) with a retention time of 2 minutes. The results of mass spectrometry analysis (Figure 1) confirmed that the active compound is a glucose hexamer. A glucose hexamer (hexasaccharide) with a molecular weight of 996 Da would have a molecular weight corresponding with that of the compound which stimulated the germination of resting spores of *P. brassicae*. The estimated concentration of the active stimulant of resting spore germination based on the height of the glucose peak in the analysis of hydrolysate was approximately 4µM.

The LCQ mass spectrum obtained from the active fraction contained several mass peaks in the range from 145 to 1000m/z. The peaks and the range indicated a carbohydrate of 6 hexose units. Several peaks could be interpreted as characteristic of a linear polysaccharide with six glucose units, but the spectrum was more complex than would be anticipated from known fragmentation patterns (Cancilla et al., 1998). It was not possible to deduce the linkage or branching pattern from the data as it is not known if the glucose units are derivatised, with for example N-acetyl groups.

Results of these experiments confirmed that an active compound capable of stimulating the germination of resting spores of *P. brassicae* and resulting in the release of motile primary zoospores is produced by roots of germinating brassica seedlings. This signalling molecule has now been identified as having a molecular weight of approximately 1 kDa and being composed of glucose subunits forming a hexasaccharide. The hexasaccharide carbohydrate identified in this research has similarities with cell wall polysaccharides (Kiely et al., 2006) which have signalling properties. But literature searches have not found reports associating hexasaccharides with root extracts from hosts of *P. brassicae*. Nor apparently, is there information regarding the importance of the molecular structure of hexasaccharides and the activation of microbial spore germination.

Understanding the chemical nature of signalling molecules in root exudates which elicit resting spore germination permits the development of additional tools for the integrated sustainable control of this pernicious pathogen. Currently there are no agrochemicals available for the control of *P. brassicae*, resistance genes are of limited occurrence and their usefulness may be eroded quite quickly by the appearance of tolerant physiological races (Dixon, 2014). Control strategies rely on soil husbandry, crop nutritional and rotational techniques. Individually none of these methods offers complete control, mitigation of damage sufficient for the culture of profitable crops comes solely from combinations of the techniques. Adding further elements into integrated control strategies increases their effectiveness and longevity as described by Rashid et al (2013). The research reported here offers a further dimension for control through the development of hexassacharide molecules into formulations which might be applied as soil or transplant applied treatments which diminish the inoculum potential of *P. brassicae* populations in infested soil. Also there are opportunities for use hexasaccharide formulations in the absence of susceptible crops thereby cleaning up land in advance of growing brassica crops.

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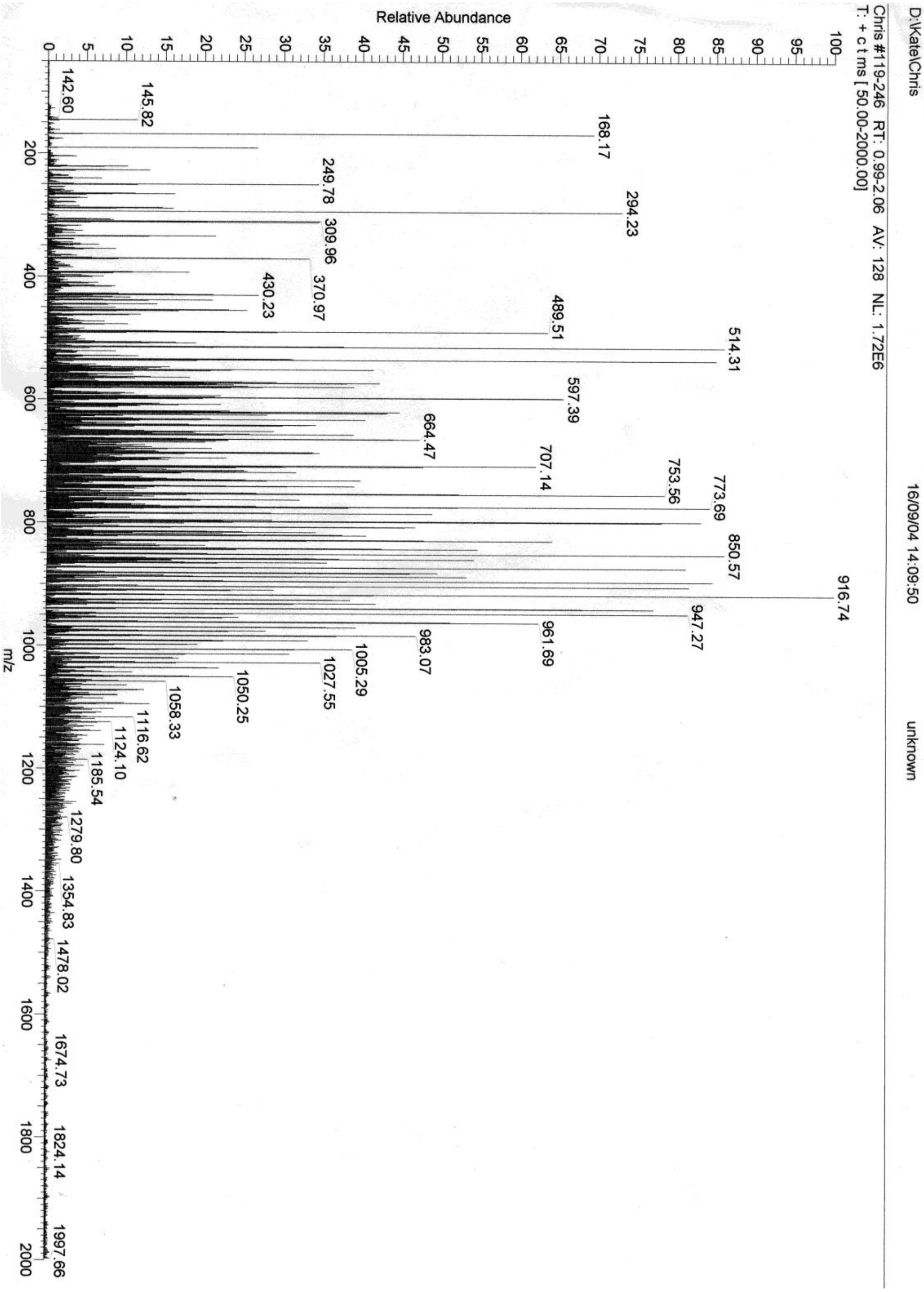
Figure 1

Mass Spectrum of the hexasaccharide which stimulated germination of resting spores of *P. brassicae*


D:\Kate\Chris
16/09/04 14:09:50
unknown
Chris #119-246 RT: 0.99-2.06 AV: 128 NL: 1.72E6
T: + c t ms [50.00-2000.00]
Relative Abundance
m/z
0
5
10
15
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45
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55
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65
70
75
80
85
90
95
100
200
400
600
800
1000
1200
1400
1600
1800
2000
142.60
145.82
168.17
249.78
294.23
309.96
370.97
430.23
489.51
514.31
597.39
664.47
707.14
753.56
773.69
850.57
916.74
947.27
961.69
983.07
1005.29
1027.55
1050.25
1058.33
1116.62
1124.10
1185.54
1279.80
1354.83
1478.02
1674.73
1824.14
1997.66