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as a means of Protecting brassica crops
from Plasmodiophora brassicae Wor.,
(clubroot)*

Article

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

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

8 Abstract

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

22 Keywords: *Plasmodiophora brassicae*, clubroot, resting spores, germination, hexasaccharide,
23 integrated control

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

34

35 The development of crop protection molecules whose mode of action operates by
36 encouraging resting spore germination offers an effective and sustainable means of control.
37 Root exudates were identified as capable of stimulating resting spore germination by
38 Macfarlane, (1970). Subsequently, Craig (1989) showed that root exudates from green
39 broccoli (*B. oleracea* var. *italica*) stimulated resting spore germination. Further research
40 demonstrated that the highest levels of resting spore germination (75%) followed treatment
41 with root exudates derived from susceptible cabbages (*B. oleracea* var. *capitata*) (Ohi *et al*,
42 2003; Hata *et al*, 2002). The research reported here describes the isolation and identification
43 of the chemical nature of specific germination stimulators derived from root exudates.

44 Materials and Methods

45 Heavily galled cabbage (*B. oleracea* var. *capitata*) roots were preserved at -20 °C until
46 required (Dixon, 1976). Resting spore extraction involved defrosting the roots, washing-off
47 residual soil and homogenising portions in 100 ml aliquots of distilled water. The resultant
48 slurries were filtered through four layers of surgical gauze and 44ml of filtrate containing

49 resting spores was decanted as 1.5ml aliquots into Eppendorf tubes. These were centrifuged
50 at 2000 g for 20 minutes. The resultant spore pellets were clarified by repeated suspension in
51 aliquots of 1ml of distilled water and re-centrifuged. Thereafter, the spore pellets were re-
52 suspended were in distilled water and stored at -20 °C.

53 The brassica host used in this research was the clubroot susceptible cabbage (*Brassica*
54 *oleracea* var. *capitata*) cv. Bartolo seed was obtained from a commercial source. When
55 required seed was germinated in Petri plates lined with surgical gauze each moistened with
56 5ml distilled water and held in darkness at 20 °C for two days and then exposed to light. A
57 sample of seedlings was used to determine that the spores of *P. brassicae* obtained by
58 centrifugation and clarification were capable of germination. These were transferred to fresh
59 Petri plates and the roots were sprayed with an aliquot of *P. brassicae* resting spores at a
60 concentration of 10^7 spores /ml as determine by haemocytometry. After 24 h root samples of
61 1 cm length were dissected and placed on a microscope slide mounted in fresh distilled water.
62 This was viewed by microscopy and showed that the resting spores had germinated and
63 released primary biflagellate zoospores which were actively swimming around the root
64 samples. This demonstrated that exudates from the roots of cv Bartolo were capable of
65 stimulating the germination of resting spores of *P. brassicae*.

66 Establishing the chemical nature of the triggers of *P. brassicae* resting spore germination
67 coming from cv Bartolo roots required increased volumes of exudates. Seed was sown into a
68 series of ten Petri plates which were prepared as described above. The resultant germinated
69 plants were allowed to grow in the plates placed in light on a north facing laboratory window
70 ledge for 10 days and water in the plates was replenished as required. At the end of this time
71 the seedlings were carefully removed from the surgical gauze and the water squeezed out into
72 a beaker using forceps. Residual moisture was present on the gauze was expressed by placing

73 it in centrifuge tubes and spinning at 2000g for 10 minutes. In total this yielded 42 ml of fluid
74 which contained root exudates produced by the germinating and growing cv Bartolo
75 seedlings. The fluid had a slightly milky appearance it was stored at 4 °C in a laboratory
76 refrigerator. It was necessary to demonstrate that this fluid contained root exudates capable of
77 stimulating resting spore germination. Approximately 0.5 ml of fluid was pipetted onto each
78 of five microscope slides and an aliquot of the resting spore suspension was added. Each
79 slide was sealed with nail varnish thereby preventing desiccation. After 20 h examination by
80 microscopy identified motile biflagellate primary zoospores of *P. brassicae* actively
81 swimming on the slide. This confirmed the presence of an active compound capable of
82 triggering resting spore germination.

83 Initial experiments with reverse phase high pressure liquid chromatography (HPLC) and
84 elution with several gradients did not find eluates with germination stimulating properties.
85 Subsequent experiments with a gravity fed gel filtration column (Sephadex 100; 120 cm x
86 1cm eluted with 0.1M phosphate buffer) recovered a compound which did stimulate resting
87 spore germination. When this stimulant was injected into the column several peaks were
88 obtained in the mass detector trace. The separation was repeated several times using a
89 fraction collector. Fractions of root exudates were collected every 4 minutes. Ultimately 52
90 tubes were collected each filled with 1 ml eluate. The potency of these fractions for
91 stimulating the germination of resting spores of *P. brassicae* was tested. An aliquot of 0.25ml
92 was taken from each fraction and placed on a microscope slide with a similar volume of *P.*
93 *brassicae* resting spores suspended in distilled water. The slide was sealed with nail varnish
94 and held at room temperature for 20hr. Microscopic examination identified the presence of
95 swimming biflagellate zoospores of *P. brassicae*. The most active fraction, number 24,
96 stimulated germination in this assay down to 10-fold dilution of the eluates from the fraction

97 collector. This positive fraction had a retention time of 85-89 minutes and a molecular weight
98 of 1 KDa. Bradford's reagent tests indicated the absence of proteins. But the large molecular
99 weight suggested that it was a carbohydrate. The active fraction was frozen and freeze-dried
100 to a white residue for mass spectral assays.

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

109 Samples from the gel column were hydrolysed with 2M hydrochloric acid 1:1 v/v at 100° C
110 for 1 hour. Calibration of the column used standard sugar samples, their retention times
111 were: mannose, 20.53; glucose, 22.65 and galactose, 24.97 respectively. The trace from a 1
112 hr hydrolysis produced a peak in the glucose position. When hydrolysis was extended to 3
113 hrs the peak reached a maximum. Three repetitions of this analysis each produced a single
114 peak in the glucose position. Mass spectral analysis was made on a Liquid
115 Chromatography Quadrupole (LCQ) Classic machine (Thermoscientific, Hemel
116 Hempstead, Hertfordshire) using an electrospray ionization needle voltage of 4.5kV and
117 capillary temperature of 250 °C. The sample was introduced in 0.1% v/v aqueous formic
118 acid and the instrument was scanned between 100 and 2,000 atomic mass units (amu) with
119 a retention time of 2 minutes. The results of mass spectrometry analysis (Figure 1)
120 confirmed that the active compound is a glucose hexamer. A glucose hexamer

121 (hexasaccharide) with a molecular weight of 996 Da would have a molecular weight
122 corresponding with that of the compound which stimulated the germination of resting
123 spores of *P. brassicae*. The estimated concentration of the active stimulant of resting spore
124 germination based on the height of the glucose peak in the analysis of hydrolysate was
125 approximately 4 μ M.

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

133

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

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

159

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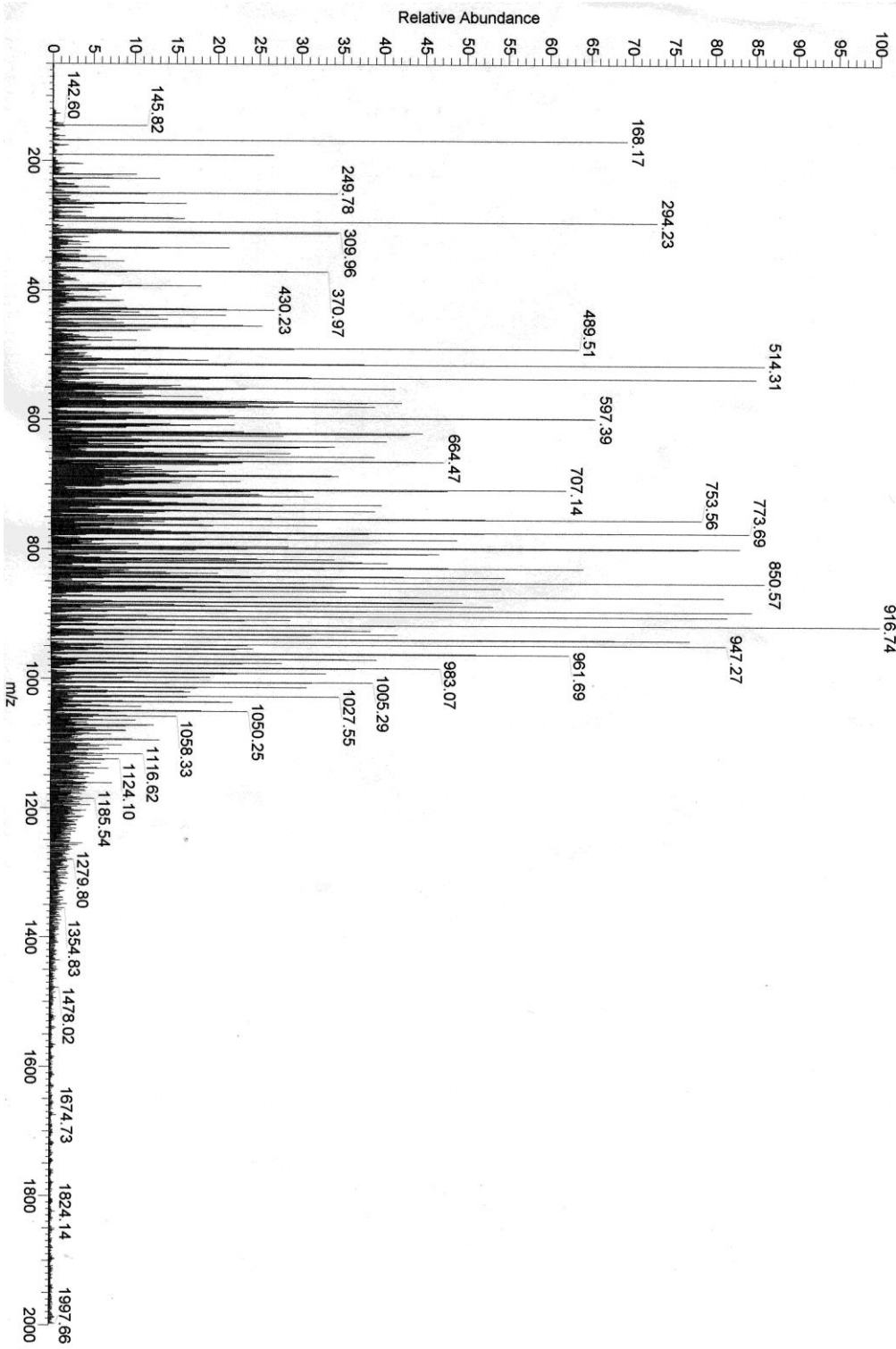
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213 **Figure 1**

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

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