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Bio-refining of perennial ryegrass (*Lolium perenne*): evaluation of aqueous extracts for plant defence elicitor activity using French bean cell suspension cultures

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Abstract

Background: There is growing interest in the bio-refining of foliage grasses to yield a range of industrial raw products. The aim of this research was to evaluate if aqueous extracts from grasses have the potential to act as crop bio-stimulants by assessing their defence elicitor activity.

Results: Field-grown foliage (500 g fresh weight) from the *Lolium perenne* varieties Tyrella, Dromara, Abermagic, and Spelga were homogenised in water (2 l) and the run-off liquid filtered through Whatman No. 1 filter paper before freezing. Thawed filtrate was centrifuged at 21,000 rpm for 15 min, to give pellet and supernatant fractions, which were assayed separately for defence elicitor activity by adding them to cell suspension cultures of *Phaseolus vulgaris* in the early exponential phase of growth (7–10 days into a 14 days culture passage). Elicitor activity was evaluated 24 h after treatment by assessing the extent of necrosis (browning) in the *P. vulgaris* cells. Supernatant and pellet fractions of all four *L. perenne* varieties were found to have potent cell defence elicitor activity, although there were relatively small but significant differences between the varieties. In a second series of extractions, homogenisation of tissues in water was compared with screw-pressed juice using the varieties, Malone, Seagoe, and Copeland. Varietal differences in elicitor activity of these extracts were removed if they were first equalised for protein content prior to eliciting the bean cells, although the screw-pressed juice was marginally more active as an elicitor than the homogenised extract. Autoclaving the extracts in attempts to solubilise/release additional elicitor compounds neither increased nor decreased the elicitor activities of the supernatant or pellet fractions. Elicitor activity of the extract was partially reduced by treating with a cation exchange resin, almost entirely removed by adsorption onto activated carbon and completely abolished by ashing at 650 °C.

Conclusion: The *L. perenne* defence elicitor(s) is therefore organic rather than inorganic, heat-stable, cationic at neutral pH, and readily soluble in water at room temperature.

Keywords: Bio-refining, Bio-stimulant, Elicitor, *Lolium perenne*, Cell suspension culture

Background

Alternative uses for forage grasses, including biomass for energy and fuels, animal feeds, in the form of protein concentrates, and various refined chemicals and fibres, have attracted much recent attention [1–3]. The social, political and environmental factors that are currently motivating this issue are complex and interrelated and have recently been reviewed [4, 5]. The process is normally referred to as the green bio-refinery or bio-refining, and usually involves the harvesting of fresh foliage or silage, which is then crushed yielding pressed juice and pressed cake that are both processed further by a variety of physical and chemical treatments, and fractionations to yield a range of products or raw materials [1,

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2, 6]. Even when all the fibre, protein or fine chemicals have been extracted, a solution, containing a wide array of dilute minerals and organic compounds, is left which is either recycled within the system or disposed of by an environmentally sustainable method such as passing through reed beds.

Here we have evaluated an alternative use for the liquid fraction of the major forage species, *Lolium perenne*, to examine if it can be used as a bio-stimulant for use on field and horticultural crops. Various naturally occurring compounds or relatively crude mixtures, with a range of bio-stimulating properties, are marketed for the optimisation of plant growth and development [7–9]. Some may provide microelements or other nutrients beneficial to plant growth. Others can act by inducing systemic acquired resistance or by containing defence elicitor activity that directly promotes defence reactions against pathogens. Many substances with stimulating and fertilising properties have been marketed as organic/mineral fertilisers and some as bio-stimulants. Indeed, Miller and Gange [10] found that over 100 bio-stimulant products were available to managers of sports turf alone. Beneficial effects of bio-stimulants on plants include increased growth, improved resistance to stress, pests and diseases, and increased water uptake [7, 11–13]. Bio-stimulants can reduce fertiliser use, improve yields, increase resistance to water and temperature stresses, and positively affects plant growth and physiology [14].

In the present study, we examine if aqueous extracts of commercial varieties of *L. perenne* can act as elicitors of plant defence using French bean cells grown in the suspension culture. This system is known to be sensitive to both endogenous elicitors [15] and elicitors from other organisms [16, 17]. Chitosan, a potent elicitor of defence reactions in plants [18], was used for comparison.

Methods

Culture of bean cells

Callus was induced on 0.5-cm long hypocotyl explants excised from sterile bean seedlings (*Phaseolus vulgaris* cv Tendergreen) on the basal medium of Murashige and Skoog [19] supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.1 mg/l kinetin and 30 g/l sucrose, with the pH set to 5.6 prior to autoclaving and solidified with 7 g/l purified agar (Oxoid Ltd. UK). Suspension cultures were prepared by adding approximately 1 g of callus to 30 ml of liquid culture medium (as for callus induction but excluding agar), in 100 ml Erlenmeyer flasks enclosed with cotton wool bungs, and shaking at 110 rpm on a reciprocating shaker. Suspensions were sub-cultured every 14 days by transferring 3 ml of cells into 30 ml of fresh medium. All cultures were incubated at 20 °C with a 16 h photoperiod provided by cool white fluorescent tubes producing 65–75 μmol/m^2/s at bench height.

To prepare uniformly inoculated suspensions for experiments, medium was prepared in bulk and inoculated with cells from 14-day-old cultures to give a density of 6–7 g/l fresh weight of cells. This bulk suspension was mixed thoroughly using a magnetic stirrer bar then dispensed aseptically into 30 ml aliquots into flasks using a sterile Eppendorf Multipipette unit.

Preparation of extracts and treatment of cell suspensions

In all experiments, bean cell suspensions were treated with *L. perenne* extracts 7 days after inoculation of the cells into fresh medium, when they were in the early exponential phase of growth. In all experiments, four replicate flasks were assessed per treatment. Foliage samples (500 g) harvested in July 2011 from field-grown plots of the *L. perenne* varieties Tyrella, Dromara, Abermagic and Spelga, were thoroughly homogenised with 2 l of water in a Waring blender then passed through Whatman No. 1 filter paper. A second series of samples collected from the varieties Malone, Seagoe and Copeland in mid September 2013, were homogenised in water as described previously, and undiluted juice was also prepared by passing foliage through a screw-press (Ponndorf Anlagenbau, GmbH). All extracts were stored at −20 °C until they were thawed and aliquots centrifuged at 21,000 rpm for 15 min to give a pellet and a supernatant. Pellets were re-suspended in a volume of water equal to that of the initial aliquot.

Comparison ryegrass varieties and supernatant with pellet fractions

The elicitor activities of either supernatant or re-suspended pellet fractions of extracts from four ryegrass varieties were tested at 3.3 or 33 ml of extract per litre of cell suspension culture. Chitosan (448877, Sigma-Aldrich, UK) treatments of 50, 100, 150 200 and 250 mg/l were also prepared in order to compare the elicitor activity with that of the perennial ryegrass extracts. An initial stock solution of chitosan was prepared by dissolving 1 g of chitosan in 100 ml of 0.5 % (v/v) acetic acid. At this concentration of acid, all the chitosan dissolved and the acid was just neutralised.

Comparison of homogenisation with screw-pressing

Extracts prepared by homogenisation in water and by screw-pressing from three ryegrass varieties were tested for elicitor activity at six treatment rates (0, 18.5, 37, 45.5, 74, 111 or 148 ml/l). All six extracts were equalised to the protein content of the lowest sample (the variety Copeland homogenised in water), as determined with the

Folin reagent, by dilution with deionised water prior to their use to elicit cells.

Effects of autoclaving extracts

Supernatant and re-suspended pellet fractions of an homogenised extract from the variety Spelga were either autoclaved (15 min at 121 °C) or non-autoclaved (control) and then assessed for elicitor activity at four treatment rates (0, 3.3, 10, 20 or 33 ml/l).

Effects treating extracts with ion exchange resins and activated carbon

Screw-pressed juice from freshly harvested Spelga foliage (May 2012) was centrifuged to produce a supernatant. Aliquots of this supernatant (18 ml) were diluted by the addition of 34.5 ml of deionised water then treated with either 1.5 g of anion exchange resin (Dowex Marathon WBA), cation exchange resin (Dowex 50wx4-400) or activated carbon (Sigma-Aldrich plant cell culture tested C6289). These mixtures were shaken for 1 h then centrifuged at 21,000 rpm for 15 min and the supernatants decanted off. The supernatant treated with the cation exchanger was adjusted to a pH of 6.80 by the addition of 0.1 M solution of KOH. Each treated extract plus an untreated control was then assessed for elicitor activity at four treatment rates (0, 10, 15, 20 or 30 ml/l).

Effect of ashing extracts

Aliquots of screw-pressed centrifuged extract from the varieties Malone, Seagoe and Copeland, equalised for protein content, were evaporated to dryness at 70 °C in ceramic crucibles and then ashed 650 °C for 17 h. The resultant ash was re-dissolved in deionised water. The pH of these solutions and comparable un-ashed controls was adjusted to 5.6 and then assessed for elicitor activity at four treatment rates (0, 33, 66 or 133 ml/l).

Assessment of elicitor activity

24 h after elicitor treatment, cellular necrosis was assessed by visually scoring the tissues for browning. A value of zero was given to tissues that remained yellowy-white, the natural colour of these cells when unchallenged by elicitors, up to a score of four for tissues that became dark brown in response to the treatments. The cells were then collected under vacuum onto 9-cm diameter Whatman No. 3 filter papers to assess their fresh weight. Protein leakage from the cells was assessed by measuring the absorbance of the culture medium filtrate at 280 nm using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK). Absorbance caused by the various extracts was compensated for by preparing controls of the extracts in water and measuring their absorbance at 280 nm.

Statistical analysis

All data were subjected to analysis of variance. Least significant difference (LSD) is shown on all figures to indicate differences between treatments.

Results

Supernatant and re-suspended pellet fractions of the aqueous extracts of all four ryegrass varieties were found to be potent elicitors of cell defence reactions, as indicated by the rapid browning of the cultured bean cells (Fig. 1a). Varietal differences in elicitor activity were evident and highly significant ($P < 0.001$) with increasing activities shown in the order Tyrella < Dromara < Abermagic < Spelga. There was also a highly significant interaction between the fraction used (supernatant and pellet) and the *L. perenne* variety ($P < 0.01$) owing to the supernatant fraction being significantly higher than the pellet fraction with the variety Dromara but not with the other three varieties.

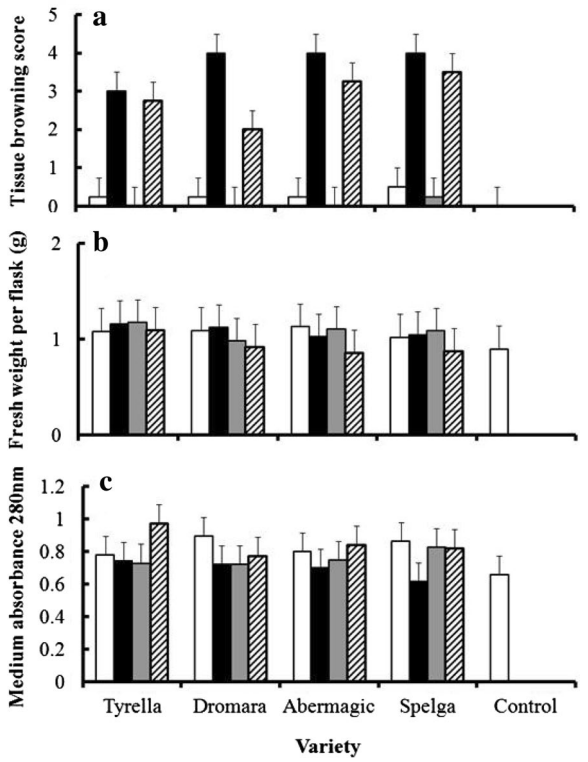

Fig. 1 Elicitation of bean cell suspension cultures with aqueous extracts from four *L. perenne* varieties, assessed 24 h after treatment, showing tissue browning score (**a**), fresh weight of cells (**b**) and culture medium absorbance at 280 nm (**c**). Suspensions were treated with either 3.3 ml/l supernatant (*open columns*) or re-suspended pellet (*shaded columns*) or 33 ml/l supernatant (*filled columns*) or re-suspended pellet (*hatched columns*). Control cells treated with water gave a score of zero on the tissue browning scale. *Bars* indicate ± the LSD at $P \leq 0.05$ (residual d.f. = 60)

The browning response was also highly dose dependant with the lower treatment rate of 3.3 ml extract per litre of cells causing little or no tissue browning (0.15 average browning score) compared to the higher treatment rate of 33 ml extract per litre of cells (2.95 average browning score). Overall, the supernatant fractions were more active ($P < 0.001$) at eliciting tissue browning than the resuspended pellet fraction giving average browning scores of 1.85 and 1.25, respectively.

The values of tissue browning obtained with the ryegrass extracts compared favourably with those obtained with the same batch of bean cells elicited with chitosan, where maximal elicitation was achieved by treatment with 50–150 mg/l chitosan (Fig. 2a). Chitosan treatments in excess of 150 mg/l produced no tissue browning. This lack of browning was indicative of cell death caused by membrane permeabilisation by the chitosan as shown by both a decline in cell fresh weight (Fig. 2b) and increased medium absorbance at 280 nm caused by leakage of proteins from the cells (Fig. 2c). In contrast, elicitation with *L. perenne* extracts had no detrimental effect on the bean cells as assessed by a decline in tissue fresh weight (Fig. 1b) and only minor leakage in a few of the treatments as indicated by medium absorbance at 280 nm (Fig. 1c).

When extract supernatants were equalised for protein content prior to their use as elicitors, no significant differences were found in elicitor activity between varieties (Table 1). However, screw-pressing yielded extracts with a significantly a higher defence elicitor activities ($P < 0.001$) from all three ryegrass varieties than did homogenisation of tissues in water (Table 1). This increased activity of the screw-pressed extracts was also highlighted by the significant ($P < 0.05$) interaction between extraction method and treatment rate caused by the screw-pressed extracts eliciting maximum tissue browning at lower treatment rates than the homogenised extracts (Table 1).

Heat treatment of the supernatant and pellet fractions by autoclaving had no significant effect on their elicitor activities, although the supernatant was again found to be significantly ($P < 0.01$) more active than the pellet (Fig. 3). The browning response was, however, highly concentration dependant with both the supernatant and pellet fractions ($P < 0.001$). Cell permeabilisation, as assessed by reduced cell fresh weight and increased medium absorbance at 280 nm, did not occur when these cells were treated with *L. perenne* extracts regardless of whether they had been autoclaved or not (data not shown).

Treatment with both of the ion exchange resins and activated carbon significantly reduced the elicitor activity of the extract indicating that they had removed components from the extract that were contributing to its elicitor activity (Fig. 4). This effect was most pronounced

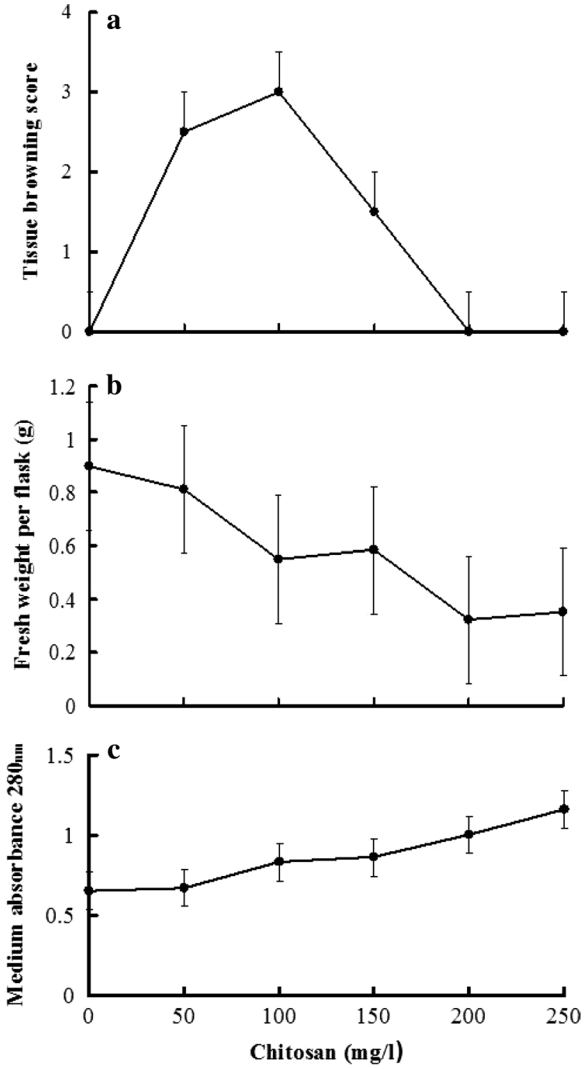

Fig. 2 The effects of chitosan concentration on the elicitation of bean cell suspension cultures. Tissue browning score (**a**), fresh weight (**b**) and culture medium absorbance at 280 nm (**c**) were assessed 24 h after treatment with chitosan. *Bars* indicate ± the LSD at $P \leq 0.05$ (residual d.f. = 60)

with treatment of the extract with activated carbon that virtually abolished the activity of the extract. The cation exchanger also adsorbed significant component of the elicitor activity, whilst the anion exchanger only had a minor effect.

Ashing the screw-pressed extracts of all three ryegrass varieties completely abolished their elicitor activity even when very high treatment rates up to 132 ml per litre were tested (data not shown).

Discussion

Potent elicitor activity, in the form of rapid browning in bean cell suspensions, was demonstrated in aqueous

Table 1 Browning scores of bean cell suspension cultures, elicited for 24 h with extracts produced either by screw-pressing or homogenising *L. perenne* foliage in water

Extraction method	*L. perenne* variety	Treatment (ml of extract per l of cell culture)[a]							Extraction method means	Extraction method × variety means	Variety means
		0	18.5	37	45.5	74	111	148			
Screw-pressing	Malone	0.50	1.00	2.00	3.00	4.00	4.00	4.00		3.00	2.81
Screw-pressing	Seagoe	0.50	1.25	1.50	3.50	3.50	4.00	4.00		2.96	2.83
Screw-pressing	Copeland	0.50	1.25	2.50	3.50	3.50	4.00	4.00		3.13	2.79
Screw-pressing (means)		0.05	1.17	2.00	3.33	3.67	4.00	4.00	3.03		
Homogenisation	Malone	0.50	1.00	1.50	2.75	3.50	3.50	3.50		2.63	
Homogenisation	Seagoe	0.50	1.25	2.00	2.50	3.50	3.50	3.50		2.71	
Homogenisation	Copeland	0.50	1.25	1.50	2.50	3.00	3.00	3.50		2.46	
Homogenisation (means)		0.05	1.17	1.67	2.58	3.33	3.33	3.50	2.60		
Treatment volume means		0.05	1.17	1.83	2.96	3.50	3.67	3.75			

ANOVA	d.f.	LSD (5 %)	Significance
Variety (V)	2	0.176	NS
Extraction method (E)	1	0.144	***
Treatment volume (T)	5	0.249	***
V × E	2	0.249	NS ($P = 0.059$)
V × T	10	0.431	NS
E × T	5	0.351	*
V × E × T	10	0.609	NS
Residual	108		

NS not significant

* $P < 0.05$, *** $P < 0.001$

[a] All six extracts were equalised to the protein content of the lowest sample (Copeland homogenised), as determined with the Folin reagent, by dilution with deionised water prior to their use to elicit the cells

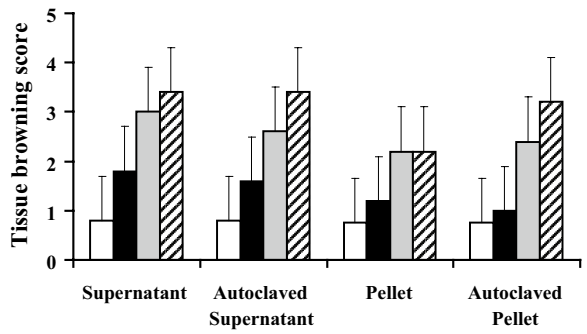

Fig. 3 Browning score of bean cell suspension cultures, elicited with supernatant or pellet fractions of aqueous extracts of *L. perenne* variety Spelga both with and without autoclaving. Assessments were made 24 h after treatment. Treatments used were 3.3 (*open columns*), 10 (*filled columns*), 20 (*grey columns*) or 33 (*hatched columns*) ml of extract per litre of bean cell suspension culture. *Bars* indicate ± the LSD at $P \leq 0.05$ (residual d.f. = 64)

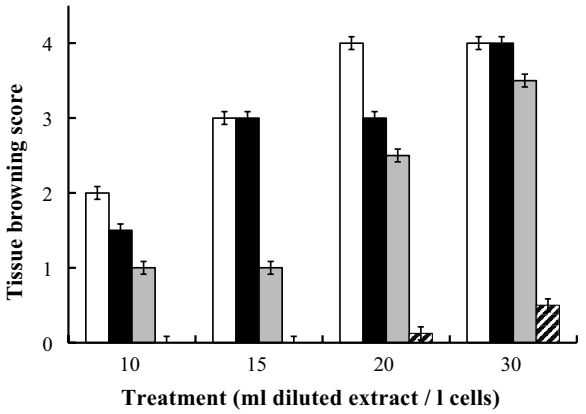

Fig. 4 Reduced elicitor activity in screw-pressed Spelga juice treated with anion exchange resin (*black columns*), cation exchange resin (*grey columns*) or activated carbon (*hatched columns*) compared to control untreated juice (*unfilled columns*). *Bars* indicate ± the LSD at $P \leq 0.05$ (residual d.f. = 48)

extracts from all seven *L. perenne* varieties examined. Browning in these cells is indicative of the general of activation phenol synthesis that includes the induction of key enzymes such as phenylalanine ammonia lyase [16], and the formation of a range of phytoalexins such as phaseolin [15, 20]. In these studies, the endogenous elicitor from 400 g [16] and 60 g [15] of bean hypocotyl tissues was used to treat one litre of cell suspension culture. In the current study, an elicitor treatment of 33 ml/l was equivalent to using 6.6 g fresh weight of *L. perenne* foliage tissues per litre of cell suspension culture, whilst lower treatment rates were also effective at eliciting cell browning (Fig. 3). Thus, the elicitor from *L. perenne* was possibly even more active than the endogenous elicitor from the bean cells themselves.

This work also indicates that an elicitor from one species can be active in a completely unrelated species. Consequently, a mass-produced bio-stimulant from *L. perenne* would be expected to have broad applicability to a wide range of crops, particularly if its mode-of-action is to induce plant defences. Transferability between species is perhaps not surprising if the active components are oligosaccharides from the cell wall, since these have a degree of structural commonality throughout the Plant Kingdom. As a result, cell wall fragments from one species have been found to have profound physiological effects on completely unrelated species [21].

Heat treatments by autoclaving, acid hydrolysis, or enzymatic treatment are often used to release elicitors from plant or fungal material, although this is not always essential [22]. With the *L. perenne* elicitor, autoclaving the supernatant or pellet fractions neither increased nor decreased the elicitor activity (Fig. 3). Thus, the elicitor(s) from *L. perenne* is water soluble at ambient temperatures and heat-stable. The possibilities remain that the elicitor activity could be increased by heating in acid conditions or by treating the extract with enzymes to release increased quantities of biologically active oligosaccharides. Clearly organic component(s) of the extracts are responsible for the elicitor activity, since ashing the extract completely abolished activity. Likewise, the elicitor is readily adsorbed by a cation ion exchanger and activated carbon indicating that it carries a positive charge at physiological pHs.

The elicitor from *L. perenne* was also demonstrated to be equally effective to chitosan, a widely used defence elicitor, but did not show the same cell membrane permeabilising properties that chitosan is known to display [23]. Thus, a bio-stimulant from *L. perenne* might have greater latitude in application rates without causing damage to cells cultured in vitro or, more importantly, plants grown in a crop situation.

It is well known that chitosan can elicit the production of secondary metabolites in tissues of a wide range of plant species grown in vitro [18]. More recently, chitosan has also been used to control *Pythium* root rot in cucumbers [24], *Phytophthora* blight in peppers [25], *Botrytis* bunch rot in grapes [26] and blue mould in pears [27]. It can also stimulate the biosynthesis of artemisinin in whole plants of *Artemisia annua* [28]. In contrast, chitosan was ineffective at protecting potatoes from late blight [29]. With most of these studies chitosan was thought to be toxic to the pathogen in addition to inducing host plant defences. In reality, the use of elicitors as plant protectants is not a new idea and was suggested by Paxton as long ago as 1973 [30].

Other preparations which elicit host defences might be equally useful as chitosan in protecting whole plants from disease. Extracts from several higher plant species have been used in this way, although most effort has focussed on species that synthesise distinctive secondary metabolites or have medicinal properties, reasoning that the metabolites produced are antimicrobial [31, 32]. The study reported here suggests that it is worth re-visiting this area to investigate plants, like the easy-to-grow grasses, which are not necessarily known for their abilities to synthesise unique secondary metabolites.

Use of a diluted fraction from pressed grass juice, or green bio-refinery waste waters, as a bio-stimulant would have the advantage that they are being produced at a time of year when most crops are in full growth. This would provide a valuable additional income stream with minimal additional costs other than those of transport and application to the crop. Other industries producing waste, during the processing large amount of plant material or spent compost, are less seasonal or even out of synchronisation with the growing season. For instance, paper pulp and mushroom production is not seasonal, whilst olive processing is mostly carried out in the winter months. Regardless of this, by-products from these three industries have been successfully tested for their uses in crop production [22, 33–37].

Conclusions

Aqueous extracts of the forage grass *L. perenne* contain a potent elicitor of plant defence reactions. To take this work further, the aqueous output from various fractions of a green bio-refinery needs to be tested in whole plant systems and in crop situations to examine if dilute foliar sprays or root drenches can increase resistance to diseases or improve crop production.

Authors' contributions

EC carried out the grass extractions and CS carried out the in vito culture aspects of the study. HSSS contributed to the management, planning and writing up of the study. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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