RESEARCH PAPER

Lack of efficacy of transgenic pea (*Pisum sativum* L.) stably expressing antifungal genes against *Fusarium spp.* in three years of confined field trials

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ABSTRACT. Fusarium root rot is a major pea disease in Canada and only partial tolerance exists in germplasm. Transgenic technologies may hold promise but the economic benefits of genetically modified (GM) pea will need to surpass the regulatory costs, time and labor involved in bringing a GM crop to market. European pea (*Pisum sativum* L.) cultivars expressing four antifungal genes, *1-3 β glucanase* (G), *endochitinase* (C) (belonging to PR proteins family), *polygalacturonase inhibiting proteins* (PGIPs) (P) and *stilbene synthase* (V) have been transformed for disease tolerance and showed disease tolerance under laboratory conditions. Transgenic lines with four antifungal genes inserted either individually or stacked through crossing were tested for their efficacy against Fusarium root rot (*Fusarium avenaceum)* in confined trials over three years (2013 to 2015) in comparison with two parental German lines and three Canadian lines. Superior emergence, higher fresh weight or lower disease ratings above and below ground, of transgenic lines in presence of disease inoculum were not observed consistently in the three years of field experiments when

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compared to the parental and Canadian lines in the presence of disease inoculum. No indication of an advantage of stacked genes over single genes was observed. Most transgenic lines had lower relative gene expression in the roots than in the leaves in greenhouse trials suggesting a possible explanation for poor tolerance to Fusarium root rot. Field trials are necessary to verify the agronomic performance and ecological relevance of the promising effects detected under laboratory conditions.

INTRODUCTION

Grain legumes are valuable worldwide, for their nutritional and health benefits and contribution towards agricultural sustainability. Among grain legumes, pea (*Pisum sativum* L.) is economically important next only to soybean and bean worldwide and is mostly grown in temperate regions. Pea is the largest pulse crop in the multi-billion dollar Canadian pulse industry, grown primarily in the prairie provinces of Saskatchewan, Alberta and Manitoba, with harvested area of 1.68 M ha in 2016.[1] The ability of pea to fix nitrogen promotes environmental stewardship by decreasing fertilizer application, reducing greenhouse gas emissions while increasing nitrogen availability for subsequent crops. Development of field pea cultivars with improved yield, disease resistance, abiotic stress tolerance and seed quality have been identified as major research priorities by Canadian pulse growers.[2] However, further improvement to yield and seed quality is limited by pea diseases, particularly the fungal endemic disease complex, Fusarium root rot (*Fusarium spp.*) which could be responsible for up to 60% yield losses in pea crops commercially in Canada.[3]

Recent disease surveys in Alberta have pinpointed *Fusarium* species associated with pea root rot complex,[4] with *F. avenaceum* as the primary causal agent, whose increasing populations may have been benefitted by the crop rotations with canola.[5] *Fusarium avenaceum* isolates show genetic and ecological plasticity, occupying various ecological niches such as root tissues of legumes, head and root tissues of cereals[6] and hence, is also the causal agent for root rots associated with other common crop crops in the prairies

including canola[7] and wheat.[8] Currently, no fungicides are effective against Fusarium root rot of pea and only partial disease resistance in pea has been identified.[9] Management strategies are reliant on crop rotation, although species involved in this fungal complex can survive for several years in soil.[5] Disease resistance would have positive economic impact on the pea industry in prairies. Genetic transformation could aid classical breeding techniques, by overcoming sexual incompatibility of related species and lack of natural sources of resistance in pea. Because of its self-pollinating nature, low degree of outcrossing and low allergenicity, pea is a good candidate for genetic modification.[2,10] However, the economic benefits of genetically modified (GM) pea will need to surpass the regulatory costs, time and labor involved in bringing a GM crop to market[11] and no GM pea has been commercialized to date for disease tolerance.

Despite the effort in crop improvement via transgenic technology, there are few examples of commercially successful transgenic legumes beyond soybean.[12] Particularly in the case of pea, biotechnological approaches have been restricted to development of insect resistance traits[13,14] or drought tolerance (Kahlon et al. 2017 unpublished). Successful examples of commercial release of GM disease resistant crops in general, are rare, currently limited to the example of ring spot virus resistant papaya genetically modified with the coat proteins from mild virus strains of the pathogen inserted.[15] The lack of GM disease resistant crops could be attributed to lower level of disease resistance conferred (compared to other traits such as herbicide resistance), which is below economic threshold for producers or a

high level of resistance but only to a very specific pathogen.[16]

Several approaches have been used to engineer plants for fungal resistance (for reviews, see ref[16-18] such as introduction of resistance-genes (R-genes)(utilizing plants basal defense responses),[19] detoxification of virulence factors,[16] expression of antimicrobial secondary metabolites like phytoalexins and pathogenesis related (PR) proteins (inhibiting the pathogen's capacity to degrade polysaccharides within cell wall or RNA),[16,20] and modification of plant signaling pathways including transcription factors genetically. It is notable that for achieving enhanced diseases tolerance, working on pathogens with a wide host range (particularly for seedling infecting pathogens), have been more successful.[17] *Fusarium* spp. are often classified as hemibiotrophs because their infection pattern initially resembles that of a biotrophic pathogen (relying on living host) and gradually transitions into a necrotropic pathogen (consumer of host cells after killing them).[21] Such pathogens invade living plant cells and subvert the metabolism in favor of their own growth, hence with such specialized plant-hemibiotrophic pathogen interactions, even minor changes in either host or pathogen can upset the balance.[22] Achieving genetic resistance/tolerance becomes even more difficult when pathogens are genetic variable, as has been reported in cases of *F. graminearum*,[23] *F. avenaceum*[24] and other *Fusarium* species, because of a variation facilitated rapid evolution of resistance.[5] Often, disease resistance is a highly complex multigenic trait and thus single gene transformations may be insufficient and/or offer limited spectrum disease resistance,[25] or single gene resistance could be circumvented by a mutation reducing the function of the introduced gene.[26] Hence, stacking/pyramiding more than one gene decreases the risk of resistance development.[27] However, coordinated expression of several genes in one plant could pose an additional challenge.[27,28]

Recently, European pea (*Pisum sativum* L.) cultivars expressing four antifungal genes, *1-3 β glucanase (G), endochitinase* (C) (belonging to PR proteins family), *polygalacturonase inhibiting proteins* (PGIPs) (P) and *stilbene synthase* (V) have been transformed for disease tolerance[29-31]

The PR proteins (*1-3 β glucanase* and *endochitinase*) degrade microbial cell wall components (i.e. glucan and chitin)[32] and PGIPs can inhibit fungal endopolygalacturonases causing fungal wall degradation and plant tissue maceration[33]; both are important components of quantitative plant defense responses. *Stilbene synthase* belongs to the phytoalexins class of secondary metabolites that possess biological activity against a wide range of pathogens.[34] Transgenic plants showed enhanced tolerance to fungi in *in vitro* testing (inhibition of fungal spore germination of *Trichoderma harzianum* (T12 strain).[29] However, field trial is crucial to establish trait efficacy, especially with soil dwelling pathogens because of the complexity and high degree of temporal and spatial variation in soil based ecosystems.[35] However, the field-testing of GM crops in Germany is complicated due to non-transparent legislation of GM crops.[36] An experienced regulatory framework exists in Canada and Canada regulates products derived from biotechnology processes under "novel products". Therefore GM plants are known as Plant with Novel Trait (PNT) and regulated under the auspices of Canadian Food Inspection Agency (CFIA), Health Canada and Environment Canada.[37]

We report here three years of investigation of confined field trial of transgenic disease tolerant pea stably expressing antifungal genes single and stacked against *Fusarium avenaceum* in comparison to parental lines and well adapted Canadian pea lines. Our hypothesis was that pea transformed with antifungal proteins would tolerate Fusarium root rot better than Canadian conventional pea lines and parental lines. We also hypothesize that transgenic lines with stacked genes will have an advantage over single gene insertions in response to Fusarium root rot in the field.

MATERIALS AND METHODS

Plant Material

Four antifungal genes V, P, G, C encoding for disease resistance were transformed into European pea cultivars "Baroness" and "Sponsor" at Institute of Plant Genetics,

Department of Plant Biotechnology, Leibniz University Hannover, Germany. Embryo axis excised from mature seeds using the modified protocol by Schroeder *et al.*,[38] of European pea cv. Baroness (*P. sativum* L. cv. Baroness) for transformation with V, P, G genes (V had inducible Vst promoter, P, G had constitutive D35S promoter), and similarly, European pea cv. Sponsor (*P. sativum* L. cv. Sponsor) was employed for transformation with C gene (promoter D35S), served as explants for *Agrobacterium*-mediated transformation using *Agrobacterium* strain EHA105.[39] Herbicide resistant *bar* gene from *Streptomyces hygroscopicus* was present along with the genes as selectable marker[40,41] (for functional map of each transgene, details of choice of promoters, sources of genes, please refer to.[42] Two single *Chitinase* gene lines, have either D35S (double 35S promoter from CaMV {Cauliflower Mosaic Virus}) (line 18) or *Vst* (*Stilbene synthase*) promoter from grape (line 20). Conventional breeding was employed to incorporate genes into a single line (V:P[43] x G:C[29] = V:P:G:C.[44]

Gene Expression

To determine if gene expression in transgenic and non-transgenic lines used in the field experiment was maintained in subsequent generations, they were grown in a greenhouse in a separate experiment. Plants (10 plants line^{-1}) were seeded in pre-autoclaved vermiculite-perlite mixture (Sunshine Mix®#4, Sun Gro Horticulture, Canada) and retained at $25 \pm 2°C$ with a 16/8-h light/dark photoperiod for four weeks after which root and leaf samples were collected and cleaned thrice with RNAse free water. According to manufacturer (Qiagen, Canada)'s recommended protocol, total RNA was extracted from tissues using the Qiagen RNeasy Plant Mini Kit. Briefly, frozen tissues were ground into a fine powder with liquid nitrogen using baked ($250°C$, 3 hr) and chilled ($-80°C$) mortar and pestle. Then a 100 mg of the powder was taken and mixed with 450 μl of buffer RLT (containing β-ME) and vortexed to obtain slurry, incubated at $56°C$ for 3 min, passed through the QIAshredder column and

centrifuged at 21,000xg for 2 min. In a collect tube (with flow through), 200 μl of absolute ethanol was added and the mix applied to RNeasy column and centrifuged at $10,000 \times g$ for 30 sec. The flow-through was dispensed off and column washed by adding 350 μl RW1 buffer, centrifuged again and subjected to on-column DNA digestion using RNase free DNase set (Qiagen) by adding 27.27 U DNase in 80 μl RDD buffer to the column and incubated at room temperature for 15 min. This was followed by additions of 700 μl RW1, 500 μl RPE and 50 μl RPE and centrifugation at 10,000xg and elimination of the flow-through to the column at each step. In a new 2 ml collection tube, the column was centrifuged at 12,000xg for 2 min and finally, transferred to a 1.5ml microfuge tube. Addition of 50 μl of nuclease free water to the centre of the column, and centrifugation of RNA eluted at 12,0000xg for 30 sec was next. A NanoDrop^{TM} spectrophotometer (Thermo Fisher Scientific^{TM}) was used to quantify the extracted RNA and it was stored at $-80°C$ until further analysis.

cDNA Synthesis

In a 20 μl volume, complimentary DNA (cDNA) was synthesized using 1 μg of total RNA using RevertAid RT kit (Thermo Fisher Scientific^{TM}) as per the recommended protocol. Briefly, 1μg of total RNA was used as the template in a 20 μl reaction containing 100 nmole of random hexamer primer, 20 U/μl of Ribo-Lock RNase inhibitor, 10 nmole of dNTP and 200 U/μl of RevertAid reverse transcriptase in 1x reaction buffer. The contents were mixed, centrifuged and incubated at $25°C$ for 5 min, followed by 60 min at $42°C$ for cDNA synthesis followed by heating the tubes to $70°C$ for 5min to inactivate the enzyme. Products were stored at $-80°C$.

Quantitation of Gene Expression by Real-Time PCR

Gene expression levels (of V, P, G, and C) in transgenic pea line's tissues (from root and leaf),

were quantified using SYBR Green based q-RT-PCR on a StepOnePlus^{TM} instrument (Applied Biosystems$^{®}$, Canada) with quantitation employing $\Delta\Delta C_T$ method with melt curve. A 10 μl reaction contained 5 μl of 2 x KAPA SYBR$^{®}$ Fast Master Mix (Kapa Biosystems, Boston, MA, USA), 1 μl of 1:15 diluted cDNA, and 5 pmol of each (forward and reverse) gene specific primers which were designed using either Primer Express 3.0 (Applied biosystems) or PrimerQuest (Integrated DNA technologies, Coralville, Iowa) with Tm of 60°C and amplicon sizes between 100-140bp. Elongation factor 1a was used as endogenous control. Primers used were "P" forward: 5′-CTTCGAAATCAAGACAGCCTTCA-3′; reverse: 5′-GGGATCACACTCGACGCAG TA-3′; "V" forward: 5-AGAAATGCCCGGTG CAGAT-3′, reverse: 5′-TTCCACCTGCATAG-CAACCTT-3′; "G" forward: 5′- AAC GCG CGG AAC TAC AA -3′, reverse: 5′- CTC GTT GAA CAT GGC GAA TAT G -3′; "C" forward: 5′- GAA CCG AAA CTC CTT CTA CAG -3′, reverse: 5′- TCC TGC TTC TTG GTG GTG -3′ and endogenous control forward: 5′-GATG-GATGCTACCACCCCTAAG-3′, reverse: 5′-GAGATGGGAACGAAGGGAATT-3′). Every reaction was carried out in triplicate using 6 cDNA samples from individual plants from each line and the average C_T values were used for calculating gene expression. The detection limit for the plasmid copies was obtained with a dilution series between 10^7 and 10^1 copies per reaction, a linear range of detection was established and were added to the German parental lines (Sponsor and Baroness)'s cDNA sample to serve as the baseline for estimating relative expression. These methods are previously published.[42]

Field Trials

A confined field trial was established at a secure field site located at the Crop Diversification Center (CDC) North, Alberta Agriculture and Forestry (AAF), north east of Edmonton, AB (lat. 53°38′N, long.113°22′W), on a black chernozemic sandy loam soil in spring of 2013, 2014 and 2015, following the regulatory guidelines for field testing of PNTs outlined by the CFIA. Seventeen treatments, comprised of nine transgenic lines (five lines with single gene insertions {5(G), 18(C), 20(C), 21(V) and 23 (P)} one line with double gene insertion {4(V: P)}, one line with triple gene insertion {11(P: C:G)}, two lines with four gene insertions {8 (V:P:G:C), 10(V:P:G:C)} and as comparator, four lines including two German parental lines, Sponsor and Baroness and three Canadian lines with/without disease inoculum : Agassiz ({resistant to Powdery mildew (*Erysiphe pisi* Syd.) and moderately susceptible to Mycosphaerella blight (*Mycosphaerella pinodes*) }[45]), AC Earlystar ({Resistant to powdery mildew moderately resistant to Mycosphaerella blight and Fusarium wilt (*Fusarium oxysporum*)}[46]) and AAC Royce ({resistant to powdery mildew, moderately susceptible to Mycosphaerella blight and Fusarium wilt[47]). Seeds were individually planted by hand at 30 seeds plot^{-1} (1 x 0.5m) at 5cm depth. Each plot was separated by seeded rows of conventional AC Ultima triticale to better delineate one genetic composition to another and limit tangling of pea lines between plots. All plots were inoculated with *Rhizobium leguminosarum* bv. Viciae (1.6 × 109 viable cells g^{-1}) (Galloway seeds Ltd. Fort Saskatchewan, AB) at a rate of 291.58 g ha^{-1} @0.004g for 2.5g seeds for promoting root nodulation. All transgenic lines, German parental controls and three Canadian lines were also treated with *Fusarium avenaceum* inoculum @5g1m^{-1} row, ground into fine power from previous year's infected wheat plants and applied in contact with the seed at the time of seeding to promote disease establishment. After soil testing each year, appropriate fertilizers were added and plots were hand weeded throughout the growing season. The plots were arranged in randomized complete block design with pea lines as treatment randomly arranged in blocks, six replicates per treatment.

Confirmation of Pathogen Presence/ Disease Verification

Pea roots, the *Fusarium avenaceum* inoculum and random soil samples from the field site for 2013, 2014 and 2015 trials were used for

characterization of the pathogen using plating and polymerase chain reaction (PCR).

Inoculum Plating

Approximately 1mg of ground *Fusarium avenaceum* inoculum used in the field experiment each year was plated onto potato dextrose agar (PDA) with antibiotics on petri dishes. Plates were incubated for 7-10 days at room temperature and resulting cultures were sub cultured and confirmed as *F. avenaceum* based on the colony morphology using culture identification techniques outlined in.[48]

Root Sample Plating

Five random roots plot^{-1} were selected in each replicate. Tap roots showing necrosis were cut into 1cm pieces and 3 pieces plot^{-1} were randomly selected each field season, transferred into 15 ml culture tube and surface sterilized using 70% ethanol for 30s followed by 0.5% NaOCl (10% bleach) for 2 min, rinsed 3X in distilled water and blotted dry on sterile filter paper. Three root pieces plot^{-1} were plated on acidified potato dextrose agar (APDA) in 90 mm Petri dish and incubated on the laboratory benchtop for 7 -10 days (8 h light, 16 h dark, 22°C). Colonies growing from the root pieces were transferred to a new APDA plate, using hyphal tip transfer under a dissecting microscope. Presumptive identification of *F. avenaceum*, *F. solani*, *F. redolens* and *F. oxysporum* were then made based on the distinct morphological characteristics of these species (pink/yellow/dark red/or purple pigmentation and shape of macroconidia under microscope) when possible, and in comparison to stock cultures as per,[48] and confirmed with PCR. Number of roots plate^{-1} yielding a *Fusarium* culture was recorded. In 2014 and 2015, the symptomatic areas of roots were also used in a series of multiplex PCR reactions to assess for presence of 10 *Fusarium* species and *Aphanomyces euteiches* (another pathogen commonly associated with root rots in prairies). Briefly, root samples were lyophilized for 48 h (4.5 L FreeZone, LabConco, Kansas City,

Missouri, USA) and then 30 mg of tissue transferred to collection microtubes in a 96-well plate format. Samples were then ground using a TissueLyzer II (Qiagen, Carlsbad, California, USA) and DNA extracted using the PlantDNA-Biosprint kit according to manufacturer's instructions (Qiagen, Carlsbad, California, USA). The multiplex reactions were performed using the Qiagen Multiplex PCR Kit according to manufacturer's directions with 2 μL of DNA and 0.2 μM of each primer (details of primers included in supplementary online material). Multiplex combinations were as follows: 1) *F. graminearum*, *F. poae*, *F. oxysporum* and *A. euteiches*; 2) *F. acuminatum*, *F. solani* and *F. equisiti*; 3) *F. culmorum*, *F. redolens*, *F. sporotrichoides* and *A. euteiches*; and 4) *F. avenaceum* (2 primer pairs). A positive DNA standard from stock cultures of all species was included with all multiplex reactions to ensure there was no cross-reactivity between primer pairs. There was cross-reactivity between the *F. avenaceum* primer pairs with *F. acuminatum* DNA. A combination of the two *F. avenaceum* and *F. acuminatum* primers could usually differentiate between these two species, but in some cases the reaction was scored as mixed for *avenaceum/acuminatum* when results were not clear.

Soil Sample Plating

Soil samples were collected from randomly chosen plots immediately prior to seeding of the trials each year. Soils were diluted 1:50 in sterile distilled water and 1ml was plated onto potato dextrose agar (PDA) with antibiotics on petri dishes. Plates were incubated for 7-10 days at room temperature and the resulting mixture of cultures were scraped from agar surface and subjected to DNA extractions and a multiplex PCR, as described above.

Plant Growth Assessment and Disease Ratings

Data was collected for seedling emergence (14 days after planting (DAP)), plant heights

TABLE 1. Above (adapted from[52]) and below ground disease rating scale (adapted from[50]).

Tissue used	Observations recorded			Rating
Above ground	Healthy plant			1
	Slight yellowing of lower leaves			2
	Yellowing of lower leaves upto the 3rd or 4th node, some stunting			3
	Necrosis of at least half or more of the plants with some stunting			4
	All plants dead or nearly so			5
Below ground	*Lesions*	*(%) Root discoloration*	*Root mass reduction*	
	0	0	0	1
	0.1-0.2cm, small reddish brown at hypocotyl base	0	0	2
	Coalescing of localized root/hypocotyl lesions from 0.5-1cm, around the stem	10-20%	0	3
	Lesions extending and completely encircling the stem	95%	5-10%	4
	Increasingly discolored and extended hypocotyl lesions	100%	20-50%	5
	Hypocotyl lesions encircling the stem extending up to 2 cm	100%	50-80%	6
	Pithy or hollow hypocotyl with very extended lesions	Dead	Dead	7

and root diameters (28, 42 DAP, 5 plants plot^{-1}). The disease symptomology and severity for above[49] and below ground plant parts[50] (Table 1) were recorded from all plants in each plot after destructive sampling at 8 weeks after planting. Fresh weight (gm plot^{-1}) was recorded as an indicator of the potential yield, because of the destructive nature of sampling for disease severity ratings.

Statistical Analysis

Data generated from field experiment were analyzed using analysis of variance (ANOVA) using PROC MIXED in SAS 9.4,[51] where lines and years were used as fixed effects and blocks as the random effect. LSmeans were compared using pre-planned orthogonal contrasts statements.

RESULTS

Gene Expression Analysis

The relative gene expression was higher in leaf tissue than in root tissue in all of the transgenic pea lines except C, where expression was similar (Table 2, previously published, in part.[42] Gene expression between genes and lines was highly variable. Relative V expression was significantly higher ($p < 0.05$) in leaves compared to roots for line 21(V)

TABLE 2. Relative gene expression ± standard error (SE) of each gene in roots and leaves, for each transgenic line used in all field experiments.

Gene		Relative gene expression	
Line		Root ± SE	Leaf ± SE
V	4(V:P)	16.87 ± 2.45	44.42 ± 4.40
	21(V)	32.85 ± 2.36	558.48 ± 84.99
	8(V:P:G:C)	0.02 ± 0.00	1.17 ± 0.35
	10(V:P:G:C)	40.47 ± 12.54	699.14 ± 220.76
P	4(V:P)	268.79 ± 159.53	32963 ± 14166.04
	23(P)	*	*
	11(P:C:G)	122.66 ± 69.40	11892.53 ± 6171.69
	8(V:P:G:C)	3.58 ± 0.47	469.48 ± 93.35
	10(V:P:G:C)	614.05 ± 72.30	111577.50 ± 27728.24
G	5(G)	127.21 ± 71.77	452.07 ± 214.25
	11(P:C:G)	1.53 ± 0.40	4.26 ± 1.03
	8(V:P:G:C)	0.32 ± 0.20	1.77 ± 0.32
	10(V:P:G:C)	94.94 ± 31.61	1566.15 ± 462.75
C	18(C)	18.01 ± 2.56	14.62 ± 4.01
	20(C)	0.10 ± 0.02	0.19 ± 0.03
	11(P:C:G)	0.25 ± 0.04	0.08 ± 0.01
	8(V:P:G:C)	0.24 ± 0.03	0.10 ± 0.01
	10(V:P:G:C)	0.08 ± 0.01	0.09 ± 0.01

*data not available

(p = 0.0011) and line 10(V:P:G:C) (p = <0.0001). It was highest in line 10(V:P:G:C) followed by line 21(V) and line 4(V:P), and negligible in line 8(V:P:G:C). Interestingly, P had significantly lower relative root P expression than in leaf, for line 10(V:P:G:C) (p = <0.0001). Highest relative P expression was observed in leaf tissue of line 10(V:P:G:C) followed by line 4(V:P), 11(P:C:G) but lower in roots of these lines and negligible in root tissue of line 8(V:P:G:C). Relative G expression of root tissue of line 10(V:P:G:C) was significantly lower than leaf tissue (p = <0.0001) which is a four gene lines, but there was no significant difference among leaf and root tissue was recorded for the four genes stacked line 8 (V:P:G:C) and three genes stacked line 11(P:C:G) as well as single gene line 5(G). All lines tested for relative expression levels of C were not significantly different between leaf and root tissues, however, line 18(C) had some low, relative gene expression (14.62 ± 4.01in leaf and 18.01 ± 2.56 in root) in comparison to negligible expression in stacked lines like 8(V:P:G:C), 10(V:P:G:C) and 11(P:C:G) and same gene but under a different promoter line 20(C). Variable relative gene expression among various genetic compositions of pea lines suggests gene silencing or possibly unequal efficacy of promoters. All transgenic lines were confirmed for homozygosity (data not shown).

Confirmation of Pathogen

In 2013, the majority of cultures resulting from the root samples were identified and confirmed by PCR as Fusarium spp. The most prevalent Fusarium species identified were *Fusarium solani, F. avenaceum* and *F. redolens* while other Fusarium species identified (for example, *F. acuminatum*) were relatively less abundant. Presence of other common soil inhabiting fungus like *Rhizoctonia* spp., *Rhizopus* spp., *Trichoderma* spp. and *Clonostachys rosea* were also recorded. The soil plating followed similar trends and *F. redolens, F. solani* and *F. avenaceum* were identified as the predominant *Fusarium* species, present in the soil. In 2014 and 2015, *F. redolens* and *F. solani* were the most abundant in soil samples with

lesser *F. avenaceum* present than in 2013. The root plating and PCR confirmations for 2014 and 2015 resulted in fewer *Fusarium* spp identified from all samples than in 2013. For 2014 samples, the major *Fusarium* spp. identified and confirmed were *F. solani, F. equisiti, F. oxysporum* and *F. avenaceum.* For 2015, the pathogens present were characterized as *Fusarium* spp. and were not found in all the samples tested.

Growth Assessment

The precipitation accumulated during the growing period for three years, at the trial location was 58.43%, 75.59% and 60.17% of the long term average (LTA) for 2013, 2014 and 2015 respectively (Table 3). The percentage emergence of plants plot^{-1} 14 DAP are summarized in Fig. 1. The line*year interaction was significant (p < 0.0001) at 0.05% level of significance and hence data is presented per year. Overall, all transgenic, parental and Canadian lines which received *F. avenaceum* inoculum showed reduced emergence (%) plot^{-1} than the Canadian lines that did not receive the inoculum which indicates that disease inoculum challenged pea seedlings.

In 2013, transgenic lines were significantly different from German parental lines 24 (Sponsor) and 25(Baroness) (p = 0.0095) and Canadian lines (with inoculum), 26(Agassiz), 28(AC Earlystar) and 30(AAC Royce) (p = <0.0001), the highest emergence (%) being for lines 23(P) & 21(V) of the transgenic lines. However, this trend did not continue in 2014 and 2015 where transgenic lines were not significantly different than the parental lines 24 (Sponsor) and 25(Baroness) and Canadian lines

TABLE 3. Precipitation at field trial location conducted in three sequential years.

| | ———mm——— | | –%– |
Location	Accumulated from May-October	Long term Average (LTA)	% of LTA
2013	201.3	344.5	58.43
2014	260.4	344.5	75.59
2015	207.3	344.5	60.17

FIGURE 1. Emergence percent (plot^{-1}) in presence or absence of disease inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trial. * Disease inoculum was not provided.
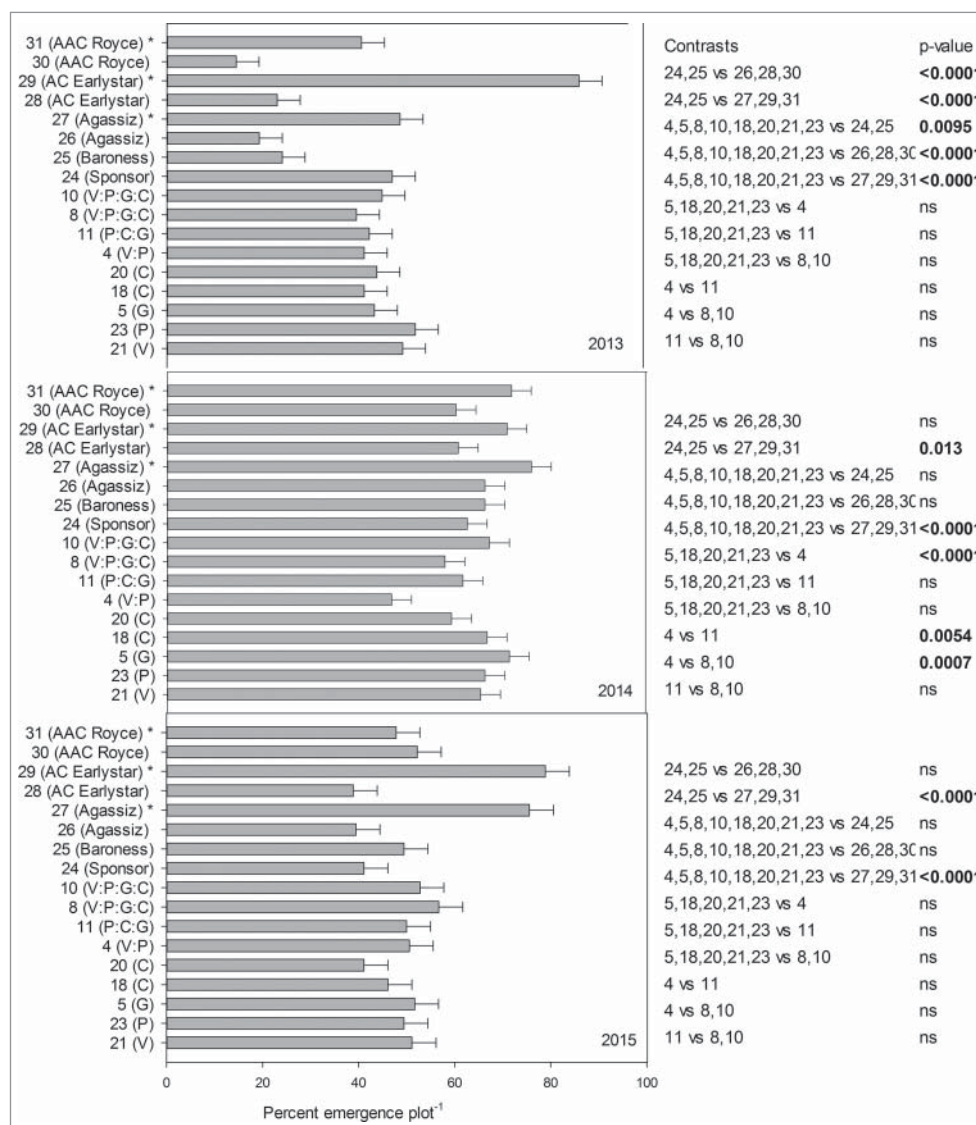
(with inoculum), 26(Agassiz), 28(AC Earlystar) and 30(AAC Royce). We observed significant differences among some genes in 2014, for instance, two genes stacked line 4(V:P) had significantly lower percent emergence than three genes stacked line 11(P:C:G) (p = 0.0054) and four genes stacked lines 8 and 10(V:P:G:C) (p = 0.0007). In 2015, the highest emergence percent in presence of disease was recorded with transgenic line 8(V:P:G:C) but it was not significantly different from parental lines 24(Sponsor) and 25(Baroness) and Canadian lines (with inoculum), 26(Agassiz), 28 (AC Earlystar) and 30(AAC Royce). In conclusion, we did not observe consistent pattern of superior emergence of transgenic line(s) in presence of disease in the three years of field experiments.

Pea plants were tallest (height cm) in 2013 followed by 2014 and 2015 but not statistically significant and no stunting or dwarfing was observed in plants that received inoculum compared to those that did not (data not shown). Because of the destructive nature of the experiment and as an indication of yield, fresh weight (gm plot^{-1}) was recorded in three years and all lines, years and line*year interaction were significant (p = <0.001) at 0.05% level of significance, therefore, data is presented separately for each year. Fresh weight (gm plot^{-1}) production was highest for lines 21(V) and 23(P), respectively, for 2013 and 2014 however, this pattern wasn't consistent for 2015 where line 5(G) had the highest fresh weight (Fig. 2). All transgenic lines had significantly higher fresh weight than their parents in 2013 (p = 0.007), but not in 2014 and 2015 and higher than Canadian lines that received disease inoculation in 2013 (p = <0.0001) and 2015 (p = 0.0396) but not in 2014. We observed that in presence of disease, single gene line 5(G), 18(C), 20 (C), 21(V) and 23(P) had significantly higher fresh weight than four genes stacked lines 8, 10(V:P:G:C) in 2013 (p = 0.0222) and 2014 (p = 0.0304) but not in 2015. Transgenic lines were significantly different in all three years (2013 and 2015 (p = <0.0001), 2014 (p = 0.014)) from the Canadian lines that did not receive inoculum (line 27, 29 and 30) asserting that disease inoculum was effective. In general, fresh weight production among the three years did not identify any transgenic line producing significantly higher fresh weight among others or in comparison to parents and Canadian lines in presence of disease or indicating an advantage of stacked genes over single genes. Interestingly, having same chitinases gene with two different promoters (line 18(C)(D35S) and 20(C)(Vst), did not yield any significant differences among the two transgenic lines for emergence (%) and fresh weight (gm plot^{-1}) throughout the three field seasons. Ample precipitation in 2014 possibly explains the higher emergence percent plot $^{-1}$ and higher fresh weight production plot $^{-1}$ in comparison to 2013 and 2015 which received lesser precipitation.

Disease Severity Ratings

The line*year interaction for disease severity ratings was significant (p<0.0001) at 0.05% and hence data is presented for each year (Fig. 3 (above) and Fig. 4 (below)). Transgenic lines had significantly lower above ground disease ratings than German parental lines (24 (Sponsor), 25(Baroness)) in 2013 (p = 0.0016) but not in 2014 or 2015 and Canadian lines that received disease inoculum (26(Agassiz), 28 (AC Earlystar, 30(AAC Royce)) in 2013 and 2015 (p = <0.0001) but not in 2014. No significant differences were observed among transgenic lines having single or two, three and four genes stacked in 2013, but there were some significant differences between single or multiple gene lines in 2014 and 2015. For instance, single gene lines (5(G), 18(C), 20(C) and 21(V)) had significantly lower above ground disease rating vs two gene line 4(V:P) in 2014 (p = 0.0016) and two gene line 4(V:P) (p = 0.0308) and three gene line 11(P:C:G) (p = 0.0334) in 2015.

Similar results were recorded for disease ratings below ground. Transgenic lines had significantly lower below ground disease severity ratings than German parental lines (24(Sponsor), 25(Baroness)) in 2013 (p = 0.0311), but not in 2014 and 2015 and lower than Canadian lines that received disease inoculum (26(Agassiz), 28(AC Earlystar, 30(AAC Royce)) in 2013 (p = <0.0001), 2014 (p = 0.0165) and 2015 (p = 0.0076). In 2013, no significant advantage of single vs multiple genes in transgenic lines for disease severity ratings below ground was recorded however, differences were observed in 2014 and 2015. For example, in 2014 two gene line 4(V:P) had significantly higher below ground disease ratings than single gene (line 5(G), 18(C), 20(C), 21(V) and 23(P)) (p = 0.0273) and four gene lines 8, 10(V:P:G: C) (p = 0.0254) and single gene lines 5(G), 18 (C), 20(C), 21(V) and 23(P) had significantly lower disease severity ratings than two gene line 4(V:P) (p = 0.0238) and four gene lines 8,10(V:P:G:C) (p = 0.0324) in 2015. Interestingly, throughout the three trial years (except above ground disease ratings in 2014), disease severity ratings above and below ground, were

FIGURE 2. Fresh weight biomass plot^{-1} (gm) in presence or absence of disease inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V: P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trial. * Disease inoculum was not provided.
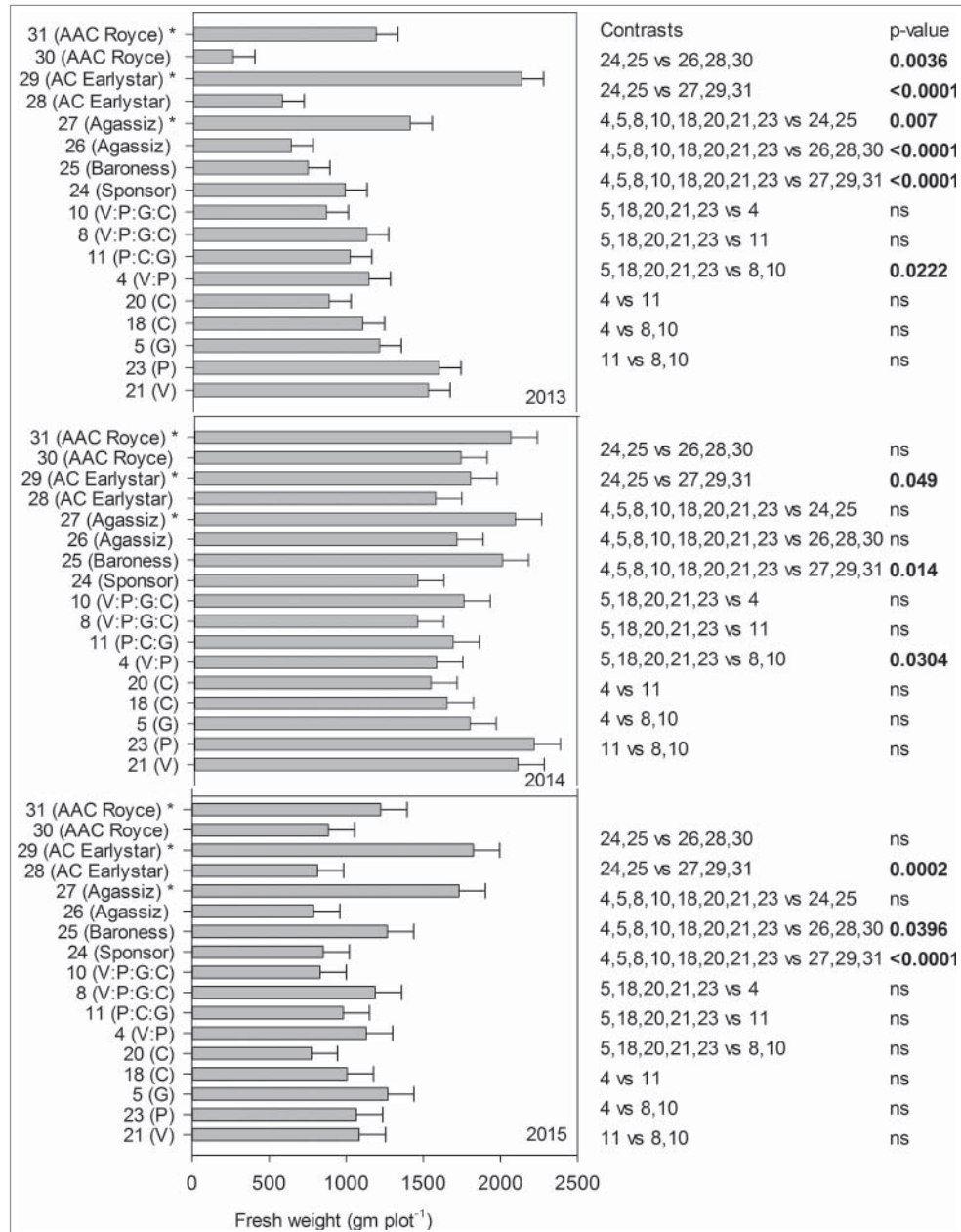
significantly lower in than the Canadian lines (26(Agassiz), 28(AC Earlystar, 30(AAC Royce)) that received disease inoculum and had some genetic advantage of partial disease resistance. It is notable that the Canadian lines have been selected in the presence (inadvertently) of similar races of these pathogens, whereas the German breeding efforts would have occurred under different selection pressure. As observed for growth parameters, lines

FIGURE 3. Disease severity ratings above ground (plot^{-1}) in presence or absence of disease inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20 (C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27 (Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trial. *Disease inoculum was not provided.
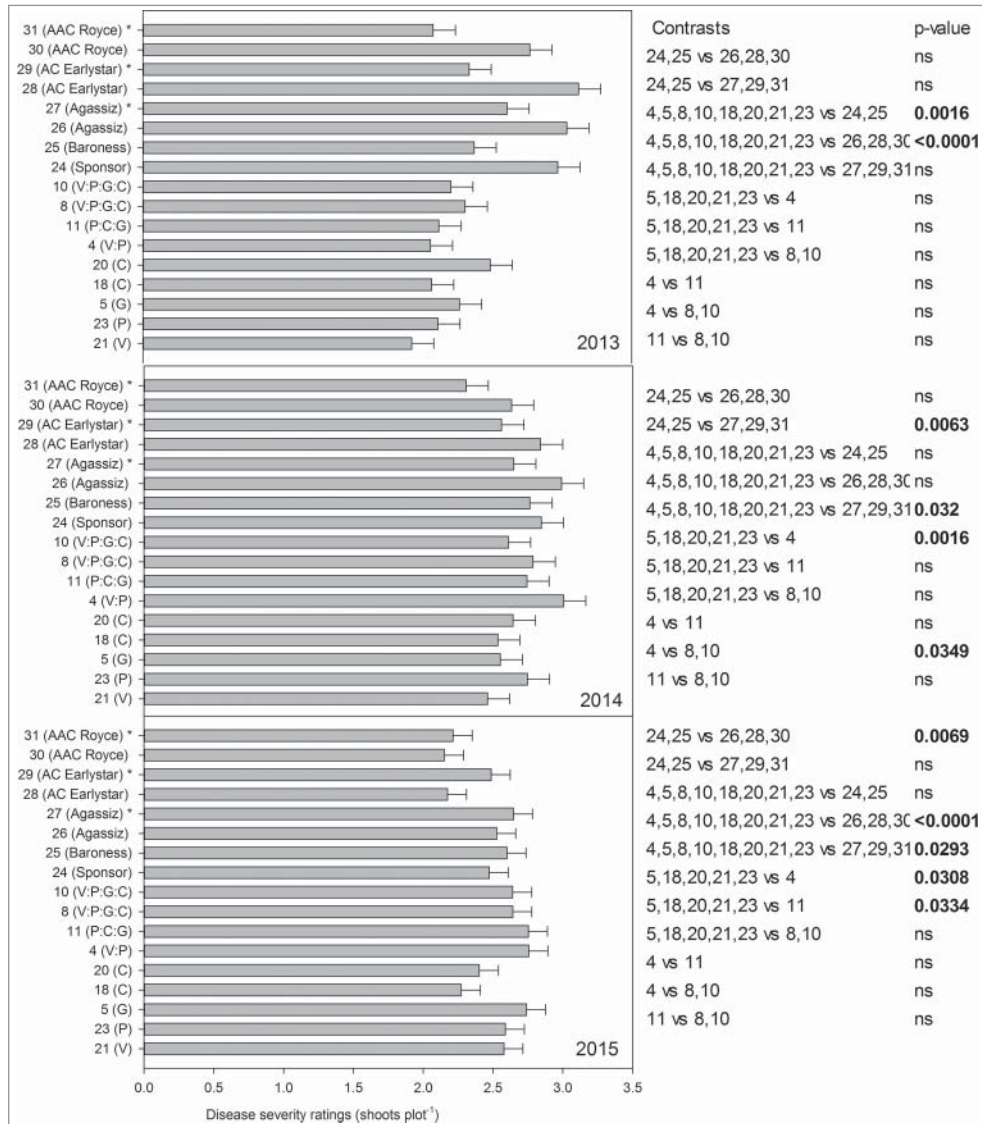

with chitinases genes with two different promoters (line 18(C)(D35S) and 20(C) (Vst), did not yield significant differences for disease severity ratings (above and below) throughout the three field seasons. Overall, contrary to our expectation, we did not observe any transgenic line or gene combination that performed better than parental lines for disease tolerance performance in three consecutive years of field trials.

DISCUSSION

In this study, confined field trials were established for three consecutive years to test nine transgenic pea lines with four antifungal genes, present singly or stacked against Fusarium root rot and were compared to their parental lines as well as Canadian pea lines in presence/absence of disease inoculum. The variability found in

FIGURE 4. Disease severity ratings below ground (plot^{-1}) in presence or absence of disease inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20 (C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27 (Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trial. * Disease inoculum was not provided.
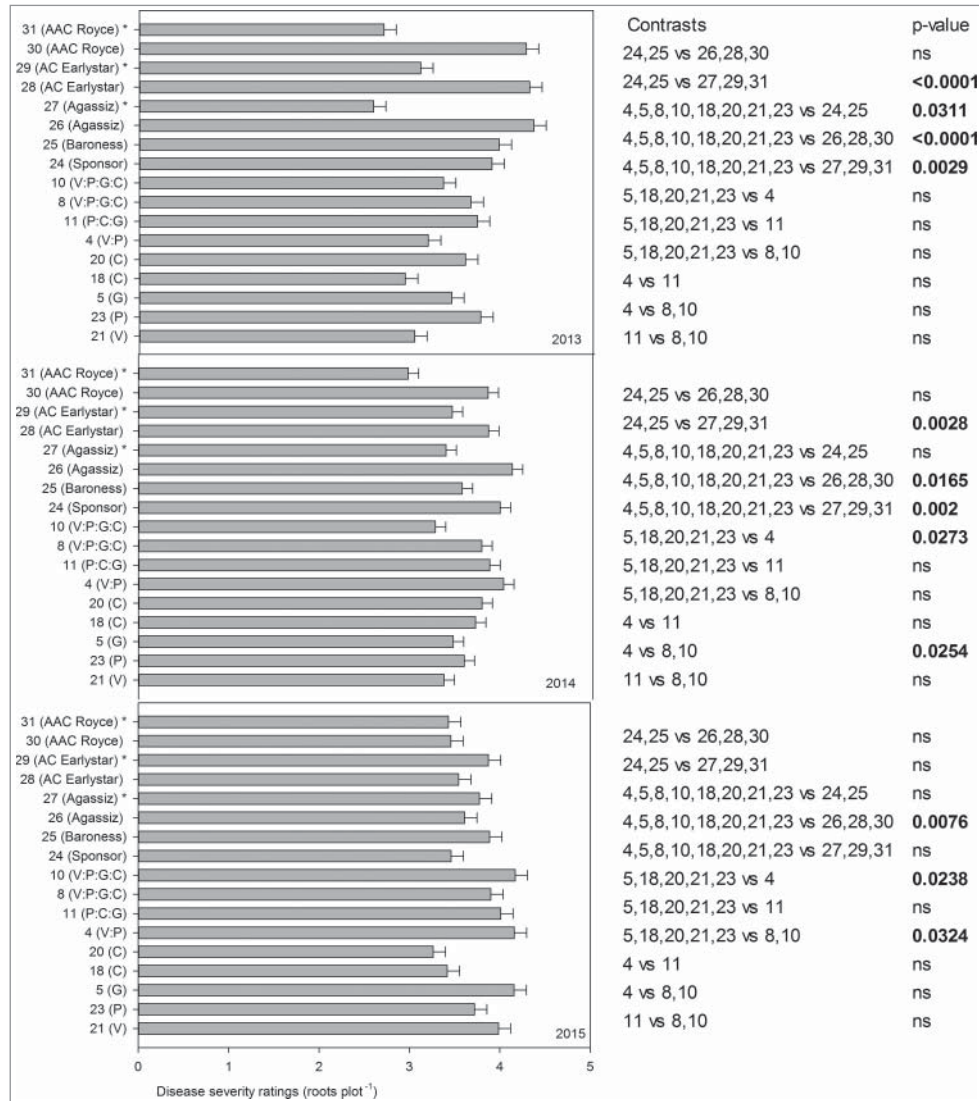
transgenic lines throughout three years of field trial reveal the complexity of the disease tolerance traits and their interaction with variable environmental conditions over the different years of the study. However, we were not able to identify transgenic pea lines that outperformed parental lines or well adapted Canadian lines in presence of disease over the course of three consequent field seasons. Although some transgenes (for example, line 21(V) and 23(P)

in 2013 and 2014) did indicate better performance (higher emergence, more biomass production and lower disease ratings) in presence of the pathogen than other transgenic lines, but that did not translate into consistent performance or differences in statistical significance vs comparators over the three trial years. No advantage of gene pyramiding over having individual genes was recorded, contrary to our initial hypothesis. Our results are consistent

with other researchers who found that transgene insertions can have variable or no effect on disease tolerance or resistance. For example, high-level expression of tobacco chitinases gene in *Nicotiana sylvestris* did not increase the resistance to *Cercospora nicotiana*,[52] bean (*Phaseolus vulgaris*) gene PGIP2 expressed in transgenic wheat did not reduce *Claviceps purpurea* symptoms[53] and *β*-1,3 glucanase constitutively expressed in alfalfa did not decrease root severity of fungi containing chitin.[54] However, contrary to what we observed, many successful transgenics, particularly in legumes, have been reported to achieving antifungal activity. For example, rice chitinases under control of CaMV35S promoter improved resistance against leaf spot (*Cercospora arachidicola*)[55] and late leaf spot (*Phaeoisariopsis personata*), rust (*Puccinia arachidis* Speg.) and *Aspergillus flavus*[56] in peanut (*Arachis hypogaea* L.). Similarly, barley chitinases (AAA56786) improved resistance to Corynespora leaf spot disease (*Corynespora cassiicola*) in Blackgram (*Vigna mungo* L. Hepper).[57] Resveratrol synthase (a stilbene) from peanut decreased Black stem and leaf spot disease (*Phoma medicaginis*) in Alfalfa (*Medicago sativa* L.).[58] Overexpression of tobacco *β*-1,3 glucanase in transgenic peanut lead to enhanced protection against *Cercospora arachidicola* and *Aspergillus flavus*[59] and *Cercospora personanta*.[60] All of these reports, however, were conducted in laboratory conditions and against single pathogen. Under laboratory conditions, differences between tested lines may be easier to differentiate as a single challenge could be imposed whereas in field trials, the number of pathogens, differences in soil moisture and weather variance more accurately represent the true agronomic effect of transgenes. Also, having multiple pathogens acting on the plant at the same time (as observed in this experiment) increase occurrence of disease symptoms.[61]

Gene pyramiding did not consistently have resistance advantages in trials conducted in the field as suggested by other researches. For instance,[62] observed no resistance to *Rhizoctonia solani* in a hyphal extension assay using extracts from potato transgenics with chitinases and glucanase gene. Transgene combinations often result in successful inhibition of fungal growth *in vitro* but fail to translate the same success in greenhouse or field conditions, as was the case of our transgenes. Oilseed rape transformed with double gene construct of chitinases and *β*-1,3-glucanase genes from barley both driven by CaMV 35S promoter did not increase fungal resistance against *Trichoderma* sp., *Alternaria brassicae, A. brassicola, Verticillium longisporum* and *L. maculans* when assayed in greenhouse whereas purified chitinases and *β*-1,3-glucanase did reduce the fungal grown in vitro.[63] This can be attributed to PR protein's differential activity against fungal cell wall targets and that they can be very specific for their resistance reaction against few pathogens yet be completely ineffective against some others in respect to same crop. This creates a challenge for continuous and sustainable resistance.[20] For example, no known PGIP is able to inhibit polygalacturonases produced by the very harmful fungal pathogen *F. verticillioides*[64] and PGIP1 which is unable to inhibit *F. moniliforme*, partially inhibits *F. oxysporum* f. sp. *lycopersici* polygalacturonases in bean (*Phaseolus vulgaris*)[65] and chitinases obtained from *Trichoderma* sp are considered more effective in conferring disease resistance.[66] The capacity of many fungal pathogens such as *Fusarium* spp, to change their genetic structure in the face of selection forces such as resistance genes and environmental factors,[17] when they possess high inherent genetic variation,[5] coupled with their hemibiotrophic nature,[21] can also contribute to difficult to achieve disease resistance. Environmental variation has been attributed to be the most important factor contributing to disease progress and resistance responses in the case of Fusarium root rot, making this trait highly challenging to accommodate.[67]

Genes used in the experiment had variable relative gene expression in transgenic lines and a lower relative gene expression in the roots as compared to the shoots in general. In particular C had very low gene expression in both root and shoot tissues, which may have contributed to a lack of resistance. No significant differences amongst single or stacked genes, or choice of promoters for the same gene were recorded.

The major constraint in co-expression of different transgenes is that the gene expression remains uncoordinated even with physically linked genes[68] and transcriptional silencing of transgene may occur. Other factors, including choice of promoters, which can affect the strength, tissue specificity, timing[69] or unexpected gene silencing[70] or transgene copy number and epigenetic effects[71-73] can contribute to varied gene expression. However, the presence of two different promoters driving the same chitinases gene (line 18 with Vst from *Stilbene synthase* and line 20 with D35SP from CamV) in the current experiment did not yield any different responses for antifungal activity.

Innumerable genes encoding for antifungal proteins have been isolated, cloned, sequenced and expressed in plants against multifarious phytopathogenic fungi with success (for a latest review, see[20]). Examples of a synergistic effect of pyramiding genes similar to what we had in this experiment on combating fungal diseases are numerous.[25,29,31,74] However, most of these results were obtained via *in vitro* and/or greenhouse testing and few crops have been released employing this strategy. Long term field testing is required to test the agronomic performance and ecological relevance of the promising effects detected under laboratory conditions in the complex environment of field especially in the local environment.[75] Field studies can help to evaluate any yield reduction which can occur due to introduction of new genes[76] or to study the interactions and interplay between various biotic and abiotic stresses in their natural form,[77] to identify and rectify issues with stability and resultant pleotropic effects[78] and to satisfy regulatory agencies who rely on results from field trials conducted at several locations and are representative of the actual target area of crop cultivation.[79] Since the goal of such research programs is improving grower yield and productivity,[80] strategic field trial experiment allowing realistic evaluation of genotype x environment interaction become crucial.

Research efforts for legume crop improvement should be encouraged as a low level of public acceptance and high costs incurred in developing and deregulating transgenic crops[11] makes it difficult for them to fit into a farmer's diversification strategy.[10] Ideally, for addressing efficacy of transgenic disease resistant plants, multiyear, multi-location field trials are desired. However, the limited seed and cost of bringing a transgenic crop like peas is beyond the budgetary scope of public institutions,[81] including ours. Often, research without significant differences between treatments are not published yet are very important for the scientific community,[82] especially considering the resources required to conduct confined release trials.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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