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Salinity drives host reaction in *Phaseolus vulgaris* (common bean) to *Macrophomina phaseolina*

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Abstract. Productivity of *Phaseolus vulgaris* L. (common bean) is often limited by diseases such as seedling blight and root and stem rot caused by the fungus *Macrophomina phaseolina* and by abiotic stresses such as salinity. This paper reports controlled environment studies examining the interaction of biotic (*M. phaseolina*) and abiotic (NaCl) stresses. Studies were conducted at 32°C. On potato dextrose agar, the growth of two isolates of *M. phaseolina* (M1, M2) was differentially stimulated by 40 mM NaCl with 1 mM $CaSO_4$. *M. phaseolina* was applied as either soil-borne inoculum or directly injected into *P. vulgaris* hypocotyls. For direct hypocotyl inoculation experiments, there was no difference in disease severity resulting from the two isolates. However, when soil inoculation was undertaken, isolate M2 caused more disease than M1. Addition of 40 mM NaCl to the soil increased disease development and severity (evident 4 days after inoculation), particularly as demonstrated in the hypocotyl inoculation tests, suggesting that salinity stress predisposes plants to infection by this pathogen. Plants infested by *M. phaseolina* showed increased tissue concentrations of Na^+ and Cl^- but decreased K^+ concentration. Hypocotyls generally contained higher Na^+ concentrations than shoots. Inoculated plants had higher Na^+ and lower K^+ concentrations than uninoculated plants. Our studies indicate that *M. phaseolina* will be a more severe disease threat where *P. vulgaris* is cultivated in areas affected by soil salinity.

Additional keywords: ashy grey stem, biotic-abiotic interactions charcoal rot, *Macrophomina phaseolina*.

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Introduction

Phaseolus vulgaris L. (common bean) is the world's most important food legume for direct human consumption, especially in Latin America and in eastern and southern Africa. Some 12 million metric tons are produced annually worldwide, of which ~8 million tons are from Latin America and Africa (FAO 2005).

P. vulgaris productivity is often limited by diseases (Schwartz and Pastor Corrales 1989). *Macrophomina phaseolina* (charcoal rot, ashy grey stem) causes seedling blight, root rot and stem rot of more than 500 cultivated and wild plant species (Sinclair 1982; Mihail and Taylor 1995; Srivastava *et al*. 2001) including common bean (Dhingra and Sinclair 1987). This pathogen is a problem in North and South America including the Dominican Republic (Sanchez 1989) and Puerto Rico (Echavez-Badel and Beaver 1987), in Asia, Africa, Europe, and also in Australia (Watson 2009). Generally, it is economically most important in subtropical and tropical countries with a semiarid climate (Wrather *et al*. 1997; Wrather *et al*. 2001) and causes severe or even complete losses in arid regions where *P. vulgaris* experiences water deficits (Mayek-Perez *et al*. 2001). In Australia, it can be a serious problem on *P. vulgaris* and commonly reduces both yield and quality, with grain yield dramatically reduced by heavy infestations of *M. phaseolina* during the reproductive phase in combination with hot and dry conditions (Redden *et al*. 1997). Thus, there is a strong association between the occurrence of drought and susceptibility to *M. phaseolina* (Gray *et al*. 1991; Manici *et al*. 1995; Mayek-Perrez *et al*. 1997).

In *P. vulgaris*, disease caused by *M. phaseolina* is characterised in young plants by black, irregular lesions which form at the base of the cotyledons and extend to the hypocotyl and stem causing strangulation and death. In adult plants, wilting and blockage of the vascular system occurs with production of black or grey microsclerotia. Salinity is one of the major factors affecting agricultural productivity worldwide, especially in the arid and semiarid areas. Even in the fertile Crescent of Jordan, Palestine, Lebanon, Syria and Iraq, and along the Nile Valley (including Egypt and Sudan), where *P. vulgaris* is a major vegetable crop, ~20–30% of the *P. vulgaris* production areas are affected by soil salinity (Bayuelo-Jiménez *et al*. 2002*b*), resulting in low yields as *P. vulgaris* is extremely sensitive to

salinity and suffers yield losses at soil salinity levels less than 2 dS m^{-1} (Läuchli 1984). The physiological responses of *P. vulgaris* to salinity stress are widely documented and vary significantly between *P. vulgaris* genotypes (Gama *et al*. 2007), when exposed to salinity at germination, seedling stage (Bayuelo-Jiménez *et al*. 2002*a*) and early vegetative growth (Bayuelo-Jiménez *et al*. 2002*b*). Salinity retards plant growth as it reduces the ability of plants to take up water, and when Na^+ and Cl^- accumulate to high concentrations in tissues this interferes with plant metabolic processes, with direct toxicity or nutrient imbalance because Na^+ competes with K^+ for binding sites essential for cellular function (Tester and Davenport 2003; Munns and Tester 2008).

The idea of developing *P. vulgaris* cultivars with resistance to both biotic (e.g. disease) and abiotic (e.g. drought, salinity) stress over a broad range of environments has been canvassed by breeders (Acosta-Gallegos 1998) and the need for selection of *P. vulgaris* genotypes with resistance to pathogens and adaptation to variable environments is now more widely recognised (Mayek-Perrez *et al*. 2003). Moreover, higher concentrations of salts in irrigation water have been linked to increased susceptibility to *M. phaseolina* of sunflower (El Mahjoub *et al*. 1979) and melon (Nischwitz *et al*. 2002), suggesting that salinity could be an important factor in the increased incidence of disease from *M. phaseolina* generally in crops.

This paper reports experiments that demonstrate salinity does increase severity of disease caused by *M. phaseolina*, and that salinity interacts with *M. phaseolina* to increase pathogen growth rate on agar plates and disease severity when in soil. This study also shows that infection by *M. phaseolina* can result in further increases in tissue Na^+ and Cl^-, and decreased K^+, in *P. vulgaris* in saline conditions.

Materials and methods

Cultivars and pathogen isolates

The *Phaseolus vulgaris* L. cultivars used were Borlotti, Brown Beauty, Gourmet Delight and Pioneer. Two isolates of *Macrophomina phaseolina* were used; these were isolated from strawberry (M1 and M2; Fang *et al*. 2011) in an area where legume crops including peas (*Pisum sativum* L.) had been severely affected by *M. phaseolina*.

Experimental conditions

Laboratory tests of growth of isolates M1 and M2 of the fungal pathogen *M. phaseolina* on potato dextrose agar (PDA) were conducted in an incubator at 32°C and without lighting. The PDA used was technical grade Difco PDA. Controlled environment experiments for plant growth were all carried out in controlled environment rooms where air temperature was maintained at 32 ± 1°C and a 12-h photoperiod with PAR of 372 µmol m^{-2} s^{-1}. This temperature was selected as it mimics that commonly experienced in the field when disease caused by *M. phaseolina* becomes evident (e.g. Fang *et al*. 2011). Plants were grown in drained pots (two plants per 20 × 18 cm pot) containing University of Western Australia potting mix (per 5 m^3 consisting of 2.5 m^3 fine composted pine bark, 1 m^3 coco peat, 1.5 m^3 brown river sand; with additional ingredients per 5 m^3 of 5 kg superphosphate, 10 kg extra fine limestone, 1.5 kg potassium sulfate, 1 kg macromin trace elements, 5 kg ammonium nitrate, 10 kg dolomite (CalMag), 2.5 kg iron sulfate (hepta) ferrous), the mix having been aerated and steam treated for 90 min at 65°C before use. All experiments were repeated at least once.

Inoculum production and use

For each isolate of *M. phaseolina*, 7-day-old colonies growing on PDA maintained at 22°C were used to inoculate millet seeds. Prior to inoculation, millet seeds were first soaked in deionised water (DI) water over night. Excess water was drained and seed autoclaved (in 250 mL flasks with ~150 mL of millet per flask for 3 × 20 min over 3 consecutive days). Autoclaved millet seeds were inoculated with the 7-day-old *M. phaseolina*-colonised PDA plugs (2 × 2 mm), mixed, and then incubated for 1 week at 22°C and shaken daily.

For soil inoculations, colonised millet seed was applied as a mixture (0.5% w/w) with potting mix and allowed to incubate for 2 weeks before sowing the *P. vulgaris* seeds.

For hypocotyl inoculations, ~0.5 g of colonised millet seeds were placed into 20 mL DI water and agitated by magnetic stirrer for 15 min to free the microsclerotia from colonised millet. Nylon mesh was used to filter out and discard the seeds. The concentration of the inoculum was adjusted to 5000 microsclerotia mL^{-1} (only microsclerotia >80 µm diameter were counted in subsamples of the inoculum using a microscope). The desired concentration was obtained by adding either more DI water or colonised millet seeds. Four days after direct sowing, each plant hypocotyl was injected with 10 microsclerotia per plant using a pipette with 10 µL disposable plastic tip and piercing directly into the hypocotyl ~0.5 mm, 2 µL of the inoculum (i.e. 10 microsclerotia $plant^{-1}$) was injected into each hypocotyl 3 cm above the ground. To ensure uniform distribution of the microsclerotia in the inoculum solution, the flask containing the inoculum was constantly shaken by hand.

Salt treatment

Salinity was imposed by watering treated pots with DI water containing 40 mM NaCl and 1 mM $CaSO_4$, whereas non-saline control pots were watered with DI water containing only 1 mM $CaSO_4$. All treatments commenced from time of sowing and continued throughout the life of each experiment. Plants were watered to free draining with the appropriate solutions daily. The potting mix contained some fertiliser (as detailed above). The same salt solutions were used for the agar growth rate tests for the two isolates (M1 and M2) of *M. phaseolina* to mimic NaCl and $CaSO_4$ conditions the fungus faced in the soil.

M. phaseolina *growth assessments*

Growth of the two *M. phaseolina* isolates was assessed at 14 h after subculturing a 2 mm plug onto the centre of freshly made PDA plates, by measuring the colony diameter with a ruler.

Disease assessments

For soil inoculations, disease assessments were made at 19 and 25 days after sowing, as this was the time normally required for symptoms to develop after soil inoculation. For the hypocotyl inoculations, disease assessments were made at 4 and 8 days after hypocotyl inoculation as symptoms normally appeared in

3–4 days. For either inoculation type, the plants were assessed for incidence/severity of disease using the same 0 to 5 scale for lesions and discoloration on the hypocotyls where: 0 = no disease; 1 = ≤1 cm lesion/discoloration; 2 = >1 to ≤1.5 cm; 3 = >1.5 –≤3 cm; 4 = >3 to ≤ 5 cm lesion/discoloration or plant collapsed from disease; 5 was where the plant had died from the infection.

Plant tissue ion analyses

Tissues were oven-dried at 60°C for 2 days, weighed, and then ground using a Sunbeam Coffee Multi Grinder (Model: EM0400). Tissue subsamples of ~0.1 g (exact weights were recorded) were extracted in 10 mL of 0.5 M HNO_3 in plastic vials placed on a shaker at 20°C for 2 days. The extract was diluted appropriately (1/100 for K^+ and 1/25 for Na^+) and then K^+ and Na^+ were measured using a flame photometer (Jenway Ltd, model PFP7). Chloride was determined using a Slamed chloridometer (Chloridometer 50cl, SLAMED Laboratory Instruments, Frankfurtam, Main, Germany). To validate the reliability of these tissue ion determinations, a certified plant tissue reference material was also analysed in the same batch as the experimental samples, with recovery being 106% for Na^+, 99% for K^+ and 103% for Cl^-. No adjustments were made to the data presented.

Experimental design and statistical analyses

There were eight two-plant replicates for each treatment (two plants per 20 × 18 cm pot) arranged in a completely randomised design, and all the experiments were repeated at least once. Analyses of variance were conducted using GENSTAT (11th edn, Lawes Agricultural Trust, Rothamsted Experimental Station, UK) to determine the effects of the different treatments and Fisher's least significant differences (l.s.d.) at $P < 0.05$ were used to test the differences between treatment means.

Results

Hypocotyl inoculation – disease severity

Salinity increased the disease severity at the first sampling time at 4 days after hypocotyl inoculation (8 days after sowing) (Tables 1 and 2). Disease severities of cultivars were different, where Borlotti had less disease. There was no difference in disease severity caused by the two isolates at the first sampling time. Cultivars Borlotti and Brown Beauty had more severe disease in the NaCl treatment than in the non-saline control. Isolate M1 with NaCl treatment caused more severe disease on Borlotti and Gourmet Delight. Pioneer suffered more severe disease from isolate M2 with NaCl than with the combination of isolate M1 with NaCl. Pioneer showed more severe disease from M2 than M1 but the other three cultivars did not show this difference towards the two isolates. Borlotti showed the most severe disease from isolate M1 in combination with NaCl. In contrast, Borlotti

Table 1. Disease severity of hypocotyl (0 to 5 scale) on four cultivars of common bean (*Phaseolus vulgaris*) caused by two isolates of *Macrophomina phaseolina* with or without soil salinity treatment (40 mM NaCl) at 4 and 8 days after direct hypocotyl inoculation (8 and 12 days after sowing) and at 19 or 25 days of growing in infested soil

Plants were assessed for incidence/severity on a 0 to 5 scale for lesions and/or discoloration on the hypocotyls where: 0 = no disease; 1 = ≤1 cm lesion/discoloration; 2 = >1 to ≤ 1.5 cm; 3 = >1.5 to ≤ 3 cm; 4 = >3 to ≤ 5 cm or plant collapsed from disease; 5 was where the plant had died from the infection

Isolate	Salinity	Cultivar	Disease severity			
			4 days	8 days	19 days	25 days
M1	40 mM NaCl	Borlotti	3.6	5.0	2.0	4.0
		Brown Beauty	3.2	4.8	3.0	3.0
		Gourmet Delight	3.0	5.0	1.6	3.0
		Pioneer	2.6	4.6	0	3.0
	Non-saline	Borlotti	1.0	4.2	1.0	3.0
		Brown Beauty	2.8	4.8	2.2	4.0
		Gourmet Delight	3.0	5.0	1.0	3.0
		Pioneer	2.2	3.8	2.0	3.0
M2	40 mM NaCl	Borlotti	2.6	5.0	5.0	5.0
		Brown Beauty	3.0	4.6	5.0	5.0
		Gourmet Delight	2.4	3.4	1.0	3.0
		Pioneer	3.2	4.8	5.0	5.0
	Non-saline	Borlotti	2.4	4.8	5.0	5.0
		Brown Beauty	2.2	4.4	2.6	4.0
		Gourmet Delight	2.8	4.8	0.6	5.0
		Pioneer	3.0	4.8	3.6	5.0

Table 2. Statistical main effects and interactions (*P*-values and l.s.d.s) from disease severity of hypocotyl (0 to 5 scale) (data presented in Table 1) on four cultivars of common bean (*Phaseolus vulgaris*) caused by two isolates of *Macrophomina phaseolina* with or without soil salinity treatment (40 mM NaCl) at 4 and 8 days after direct hypocotyl inoculation (8 and 12 days after sowing) and at 19 or 25 days of growing in infested soil

Days	4		8		19		25	
	P-value	l.s.d. at *P* = 0.05	*P*-value	l.s.d. at *P* = 0.05	*P*-value	l.s.d. at *P* = 0.05	*P*-value	l.s.d. at *P* = 0.05
Main effects								
Salinity	<0.001	0.2	–	–	–	–	–	–
Cultivar	<0.05	0.3	–	–	<0.005	1.3	–	–
Isolate	–	–	–	–	<0.001	0.9	<0.005	0.8
Interactions								
Salinity × cultivar	<0.001	0.4	<0.05	0.5	–	–	–	–
Salinity × isolate	<0.005	0.3	<0.05	0.4	–	–	–	–
Cultivar × isolate	<0.001	0.4	<0.001	0.5	<0.05	1.8	–	–
Salinity × cultivar × isolate	<0.001	0.6	–	–	–	–	–	–

suffered least disease with M1 in absence of NaCl, and there was no difference in disease severity on this variety when comparing isolate M2 with or without NaCl. Brown Beauty had more severe disease from M2 with NaCl than without NaCl (Tables 1 and 2).

There were no differences in disease severity between cultivars, isolates or salinity at 8 days after hypocotyl inoculation (12 days after sowing), by this stage all cultivars suffered disease scores above 3.5. Borlotti with NaCl treatment showed more severe disease than both Gourmet Delight with NaCl and Pioneer without NaCl. Gourmet Delight showed the least disease with NaCl treatment. Gourmet Delight showed most and Pioneer least severe disease with isolate M1. In contrast, Gourmet Delight showed less severe disease with isolate M2 than other three cultivars, whereas Pioneer had the most severe disease. There were no significant three-way interactions between cultivars, salinity and isolates (Tables 1 and 2).

Macrophomina *growth response to salinity*

M. phaseolina showed increased growth when on PDA with 40 mM NaCl plus 1 mM $CaSO_4$ or where only 1 mM $CaSO_4$ had been added, compared with growth on normal PDA. Further, growth differed between the two isolates, with M2 growing faster than M1 (Tables 3 and 4).

Soil inoculation – disease

There were differences in relation to disease severity amongst the four cultivars as well as between the two isolates 19 days after sowing in the soil inoculation test. Gourmet Delight showed the least disease severity, Borlotti and Brown Beauty showed more severe disease. Isolate M1 caused less disease than isolate M2. NaCl had no effect on disease severity. There was a significant interaction between cultivars and isolates where M2 caused more severe disease on Borlotti but less disease on Gourmet Delight. Gourmet Delight showed least disease for both isolates. There were no significant interactions between all other tested factors (Tables 1 and 2).

Isolate M2 caused more severe disease than M1 at 25 days after sowing. There were no differences in relation to disease severity between cultivars, salinity or their interactions at this time period, with all cultivars having severe disease (3–5 disease score range) (Tables 1 and 2).

Table 3. Colony growth diameter (mm) after 14 h at 32°C for two isolates (M1 and M2) of *Macrophomina phaseolina* on potato dextrose agar (PDA) with 40 mM NaCl + 1 mM $CaSO_4$ added, or with only 1 mM $CaSO_4$ added as a control comparison, or just plain potato dextrose agar

Initial colony was a 2 mm plug transferred onto each plate

Isolate	Growth (mm diameter of colony)		
	NaCl + $CaSO_4$	$CaSO_4$	Control
M1	30	29	21
M2	39	36	27

Table 4. Statistical main effects and interactions (*P*-values and l.s.d.s) of colony growth diameter (mm) (data presented in Table 3) after 14 h at 32°C for two isolates (M1 and M2) of *Macrophomina phaseolina* on potato dextrose agar (PDA) with 40 mM NaCl + 1 mM $CaSO_4$ added, or with only 1 mM $CaSO_4$ added as a control comparison, or just plain potato dextrose agarTable

Treatment	*P*-value	l.s.d. at $P = 0.05$
Main effects		
Salinity	<0.001	2.2
Isolate	<0.001	1.8
Interactions		
Salinity × isolate	<0.001	3.1

Hypocotyl inoculation – tissue ion concentrations

There were no significant effects of NaCl, cultivar, plant tissue (i.e. hypocotyl or shoot), or isolates, nor their interactions in relation to Cl^-, Na^+ or K^+ concentrations in plant tissues sampled at 8 days after sowing (4 days after hypocotyl inoculation) (data not shown).

The NaCl treatment resulted in increased Cl^- concentrations in plant tissues 8 days after inoculation. Cultivars Gourmet Delight and Pioneer had the highest Cl^- concentrations and Borlotti the lowest. Pathogen inoculation had no effect on Cl^- concentration in the plants (Tables 5 and 6).

Salinity also resulted in increased plant tissue Na^+ concentrations, and hypocotyls had significantly higher Na^+ than shoots (Tables 5 and 6, and Table S1 available as an Accessory Publication to this paper). The interaction of salinity and plant tissue type was significant; with NaCl treatment, hypocotyls contained more Na^+ than shoots. The interaction of cultivar and tissue type also was significant; hypocotyls of Gourmet Delight had the highest Na^+ concentration and the shoots of Pioneer and Gourmet Delight had the least.

In the case of K^+, salinity and inoculation individually reduced K^+ concentrations in plant tissues. The interaction of salinity and tissue type and the interaction of plant tissue and inoculation with the pathogen both had a significant but separate impact on K^+ in plants; hypocotyls treated with NaCl or inoculated with the pathogen had least K^+, whereas hypocotyls of plants without NaCl or pathogen had the highest K^+ concentrations (Tables 5 and 6, and Table S2).

Soil inoculation – tissue ion concentrations

Salinity increased plant tissue Cl^- concentrations and shoots had higher Cl^- than hypocotyls 19 days after sowing. Inoculated plants had lower Cl^- concentrations than uninoculated plants. The interaction of salinity and inoculation significantly impacted on Cl^- concentration; where plants grown in soil treated with NaCl had the highest Cl^- concentration (and the control plants had lowest Cl^- concentration). The interaction of salinity and cultivar significantly impacted on Cl^- concentration; where Pioneer without NaCl had the highest Cl^- concentration whereas Borlotti treated with NaCl had the least Cl^- concentration (Tables 5 and 6).

Salinity increased the Na^+ concentration in the plant. Hypocotyls had higher Na^+ concentration than shoots. Inoculated plants had lower Na^+ concentration. The hypocotyls of plants in soil treated with NaCl had highest Na^+ concentration and the shoots of plants in soil without NaCl the least. Plants in soil treated with NaCl but without inoculation had

Table 5. Plant tissue Cl^-, Na^+ and K^+ concentration in shoot and hypocotyl of four cultivars of common bean (*Phaseolus vulgaris*) inoculated/treated with/without *Macrophomina phaseolina* (both isolates were pooled) and 40 mM NaCl at 8 days after direct hypocotyl inoculation (12 days after sowing) and at 19 days after sowing into inoculated soil

Salinity	Cultivar	Hypocotyl		Shoot	
		Inoculated	Nil	Inoculated	Nil
8 days after direct hypocotyl inoculation					
Tissue Cl^- concentration (μmol g^{-1} DW)					
40 mM NaCl	Borlotti	595	531	744	811
	Brown Beauty	1125	861	1158	1069
	Gourmet Delight	1293	1225	1087	1037
	Pioneer	1329	1012	1179	1027
Non-saline	Borlotti	77	112	84	136
	Brown Beauty	90	109	121	138
	Gourmet Delight	107	94	107	116
	Pioneer	111	98	111	136
Tissue Na^+ concentration (μmol g^{-1} DW)					
40 mM NaCl	Borlotti	523	1095	438	540
	Brown Beauty	1246	1267	556	571
	Gourmet Delight	1957	2040	530	316
	Pioneer	1465	1301	444	338
Non-saline	Borlotti	392	393	238	471
	Brown Beauty	457	442	307	525
	Gourmet Delight	586	602	300	268
	Pioneer	359	379	271	213
K^+ concentration (μmol g^{-1} DW)					
40 mM NaCl	Borlotti	265	1630	1329	1628
	Brown Beauty	1160	2092	1764	1574
	Gourmet Delight	965	2054	2134	1570
	Pioneer	1821	1632	2002	1625
Non-saline	Borlotti	1705	1876	1559	1813
	Brown Beauty	1782	2579	1641	1692
	Gourmet Delight	1911	2003	1999	1601
	Pioneer	1306	1727	1887	1537
19 days after sowing into inoculated soil					
Tissue Cl^- concentration (μmol g^{-1} DW)					
40 mM NaCl	Borlotti	471	926	1015	1255
	Brown Beauty	550	980	926	1331
	Gourmet Delight	937	1019	973	1356
	Pioneer	NA	1144	1404	1451
Non-saline	Borlotti	67	82	137	131
	Brown Beauty	81	90	124	98
	Gourmet Delight	87	106	131	120
	Pioneer	149	169	351	172
Tissue Na^+ concentration (μmol g^{-1} DW)					
40 mM NaCl	Borlotti	793	1557	522	339
	Brown Beauty	243	1285	504	247
	Gourmet Delight	1022	1858	256	339
	Pioneer	NA	1638	673	314
Non-saline	Borlotti	221	316	274	234
	Brown Beauty	439	528	229	213
	Gourmet Delight	523	646	236	242
	Pioneer	407	521	205	224
Tissue K^+ concentration (μmol g^{-1} DW)					
40 mM NaCl	Borlotti	998	1913	1540	1528
	Brown Beauty	NA	934	1539	1560
	Gourmet Delight	1917	1950	1403	1465
	Pioneer	NA	1934	1539	1560
Non-saline	Borlotti	996	1347	1624	1660
	Brown Beauty	2002	2360	1515	1491
	Gourmet Delight	2342	2230	1538	1584
	Pioneer	1817	1913	1540	1528

Table 6. Statistical main effects and interactions (*P*-values and l.s.d.s) for plant tissue Cl^-, Na^+ and K^+ concentration in shoot and hypocotyl (data presented in Table 5) of four cultivars of common bean (*Phaseolus vulgaris*) inoculated/treated with/without *Macrophomina phaseolina* (both isolates were pooled) and 40 mM NaCl at 8 days after direct hypocotyl inoculation (12 days after sowing) and at 19 days after sowing into inoculated soil

	Tissue Cl^- concentration		Tissue Na^+ concentration		Tissue K^+ concentration	
	P-value	l.s.d. at $P = 0.05$	*P*-value	l.s.d. at $P = 0.05$	*P*-value	l.s.d. at $P = 0.05$
8 days after direct hypocotyl inoculation						
Main effects						
Salinity	<0.001	134	<0.001	191	<0.05	196
Cultivar	<0.05	190	–	–	–	–
Inoculation	–	–	–	–	<0.05	196
Tissue (hypocotyl/shoot)	–	–	<0.001	191	–	–
Interactions						
Salinity × tissue	<0.001	271	–	–	<0.05	277
Cultivar × tissue	<0.05	383	–	–	–	–
Tissue × inoculation	–	–	–	–	<0.001	277
Salinity × inoculation	–	–	–	–	–	–
Salinity × cultivar × tissue	–	–	–	–	–	–
Salinity × cultivar × tissue	–	–	–	–	–	–
19 days after sowing into inoculated soil						
Main effects						
Salinity	<0.001	21	<0.001	163	<0.005	202
Inoculation	<0.001	64	<0.005	163	<0.001	202
Tissue (hypocotyl/shoot)	<0.001	64	<0.001	163	–	–
Interactions						
Salinity × tissue	<0.001	90	<0.05	230	<0.05	285
Cultivar × tissue	<0.005	127			<0.05	404
Tissue × inoculation	<0.05	90	<0.001	230	<0.005	285
Salinity × inoculation	<0.001	90	<0.05	230	<0.05	285
Salinity × cultivar × tissue	<0.05	180	–	–	–	–
Salinity × tissue × inoculation			<0.001	33	<0.05	404
Salinity × cultivar × tissue × inoculation	<0.05	180	–	–	–	–

higher Na^+ concentrations than plants in inoculated soil. Hypocotyls of uninoculated plants showed the highest Na^+ concentration whereas the shoots of uninoculated plants showed the lowest Na^+ concentration. The hypocotyls of plants in NaCl treated soil but uninoculated showed the highest Na^+ concentration whereas the shoots of plants without both inoculation and NaCl showed the lowest Na^+ concentration (Tables 5 and 6).

Salinity and inoculation significantly and separately decreased K^+ concentration in plants. Hypocotyls of plants with NaCl treatment showed lowest K^+ and those of plants without NaCl added to the soil showed highest K^+ concentration. Hypocotyls of Gourmet Delight had highest K^+ and the hypocotyls of Borlotti had the lowest K^+ concentration. Plants in soil treated with NaCl and inoculated with the pathogen had the lowest K^+ whereas plants in soil without NaCl but with the pathogen had the highest K^+ concentration. Hypocotyls of non-inoculated plants had the highest K^+ and the hypocotyls of inoculated plants had the lowest K^+ concentration. However, shoots of inoculated and non-inoculated plants showed no significant differences in K^+ concentration. Hypocotyls of plants in soil without NaCl treatment but with the pathogen showed the highest K^+ concentration whereas hypocotyls of plants in soil without NaCl treatment but with the pathogen showed the lowest K^+ concentration (Tables 5 and 6).

Analyses of Cl^- and Na^+ in tissues sampled at 25 days (Tables S1, S3) showed that salinity increased plant tissue Cl^- and Na^+ concentrations, but differences between plants in inoculated and uninoculated soil were less evident than at 19 days (described above). Hypocotyls contained higher Cl^- and Na^+ than shoots. By contrast with the earlier sampling, at 25 days cultivar impacted on Cl^- concentration in the plants; where Pioneer had highest and Borlotti the lowest Cl^- concentration. Similarly, at 25 days, tissue Na^+ concentration also differed between cultivars; where Pioneer had highest Na^+ concentration and Borlotti the lowest Na^+ concentration. The interaction of salinity and cultivar also had significant effects on tissue Cl^- and Na^+ at 25 days. The interactions of salinity and inoculation, as well as cultivar and inoculation, were both significant for both Cl^- and Na^+ concentrations in the plant; and so was the three-way interaction between salinity, inoculation, and cultivar. These significant interactions highlight the complex nature of tissue ion regulation by the plants when under combined salinity and pathogen stresses.

Tissue K^+ data at the 25 days sampling (Table S2) showed responses in addition to those seen at the 19 days sampling

(described above). Whereas at 19 days the main effects, with the exception of inoculated versus non-inoculated, were not significant, by 25 days the main effects as well as the numerous interactive effects (of which several were seen also at 19 days) were evident. At 25 days, salinity decreased tissue K^+ concentrations, and cultivars differed in this response. Borlotti had highest K^+ concentration and Brown Beauty had the lowest. Hypocotyls had higher K^+ concentration than shoots. Plants from inoculated soil had lower K^+ concentrations compared with those from uninoculated soil. In addition, there were several interactions between the main effects of salinity, cultivar, tissue type, and inoculation, as shown in Table S2. Of these, the interaction of cultivar and inoculation significantly impacted on the K^+ concentrations in plants, so although inoculation reduced tissue K^+ the magnitude of the reduction differed between cultivars; however, no three-way interaction on tissue K^+ concentration was seen for the combination of cultivar, inoculation and salinity.

Discussion

Results of the present study showed that although there was no difference between isolates of *M. phaseolina* in terms of disease severity when making hypocotyl inoculations, there were differences between the two isolates when soil inoculation was undertaken. M2 caused more severe disease than isolate M1 following soil-borne inoculations and combined with salinity. Soil inoculation best approximates what occurs under field conditions and in the soil the pathogen was directly exposed to NaCl in the salinity treatment. The *M. phaseolina* isolates in direct contact with NaCl in the saline soil might have responded in a similar, positive way as to the NaCl treated PDA. Moreover, salt stress predisposes plants to infection by soil-borne pathogens, leading to increased root rot severity (Swiecki 1984; El-Abyad *et al*. 1988). In contrast, for hypocotyl inoculation, the pathogen was growing inside of the plant hypocotyls where the effect of NaCl would be expected to be more directly on the plant rather than on the pathogen, which was demonstrated by our two isolates showing no differences in relation to disease severity following hypocotyl inoculation. Nevertheless, as NaCl is known to affect plant cell survival, division, and growth (Blumwald 2000; Hasegawa *et al*. 2000; Zhu 2003; Munns and Tester 2008), the adverse effects on salinity on the plants could, in turn, enhance plant susceptibility to pathogen attack. In our study using hypocotyl inoculation, the interaction of NaCl and the pathogen (both isolates) significantly reduced plant growth (dry weight when 12 days old; data not shown).

The hypocotyl inoculation method, in contrast to the soil inoculation method, provides a quick, reliable and repeatable way for assessing germplasm resistance. It largely eliminates other environmental influences, for example, soil nutrient elements and pH, and other microbes in the soil on *M. phaseolina* and focuses on direct pathogen – host interactions and relationships. For hypocotyl inoculations, the fact that salinity increased disease severity as the disease was still developing (4 days after inoculation) but had no significant impact on disease severity once disease had advanced (8 days after inoculation) suggests that assessments of this biotic-abiotic interaction are best done while disease symptoms are still developing. In our study, *M. phaseolina* showed increased growth when 40 mM NaCl plus 1 mM $CaSO_4$ was added to the PDA, compared with growth on normal PDA. However, adding 1 mM $CaSO_4$ alone also improved growth, so this response may primarily have been to $CaSO_4$, but at least NaCl did not diminish this faster growth. Isolate M2 grew faster than isolate M1 on PDA and the observation that isolate M2 caused more disease than M1 in soil inoculation test may relate to M2 being able to grow and multiply faster therefore increasing the amount of inoculum for infecting plants than could M1. M2 also tended to respond more to salinity in PDA plates than M1. The work of Bouchibi *et al*. (1990), who found that salinity increased sporangium production by *Phytophthora parasitica*, supports this conclusion. Fungi have an absolute requirement for K^+, but K^+ may be partially replaced by Na^+. For example, Na^+ uptake in *Ustilago maydis* and *Pichia sorbitophila* can be rapid (Benito *et al*. 2004).

The four common bean cultivars we tested showed significantly different responses in relation to disease caused by the two isolates of *M. phaseolina*, by the saline environment and by the interaction between isolates and saline environment. For example, in soil inoculated plants, Borlotti had less disease in the early stage (8 days old) but disease progressed in the later stage (12 days, 19 days) and to where it became more diseased from isolate M2, whereas by 19 days Gourmet Delight showed a much lower disease level. In general, M2 was more pathogenic than M1 and Gourmet Delight was more robust than other cultivars tested. There are known differences between cultivars in many different crops in responding to NaCl and to pathogens, as well as in cultivar responses at different plant development stages. Natural host resistance to a pathogen is where a pathogen is less able to cause disease on one genotype or host compared with another. To this end, such resistance can be a consequence of several distinct phenomena that can operate simultaneously or at different phases of infection and disease development (Collinge *et al*. 2010). For example, resistance can be expressed at the penetration stage by determining the ability of a fungal pathogen to assimilate enough nutrients to be able to proliferate in the tissues or sporulate or spread. Host resistance can be constitutive or induced, and it has been demonstrated in several plant species that induced resistance can be regulated by different signalling pathways (Tyagi *et al*. 2008). The present study shows that host resistance expression (in terms of disease severity) is also impacted by interactive effects of salinity, at least for common bean.

Common bean in NaCl treated soil showed increased Cl^- and Na^+ concentrations (e.g. 12, 19 and 25 days old) in comparison with control plants in our study. High tissue Na^+ and Cl^- in shoots of common bean is consistent with observations (Boursier *et al*. 1987) that salt-sensitive plants generally have a poor capacity to restrict accumulation of these ions in shoots. Our study showed that plant hypocotyls generally had higher Na^+ concentration than shoots at tested plant ages of 12, 19 and 25 days. The increased ion concentrations in hypocotyls may be of consequence for disease development, as the pathogen mostly either commences at the hypocotyls or spreads towards hypocotyls and since the pathogen grows faster in a moderately saline environment at 32°C (PDA plate experiment).

Our study showed that K^+ concentration in the plants grown in NaCl treated soil and in the inoculated plants was significantly

lower than for plants grown in non-saline soil and for uninoculated plants at all tested growth stages (viz. 12, 19 and 25 days). Even in the needle inoculated treatment, inoculation reduced K^+ dramatically. However, Gourmet Delight, which presented the lowest disease scores at 19 days (Table 3), was also the only cultivar to maintain its K^+ concentration in hypocotyls under inoculation (Tables 5 and 6). K^+ is the preferred inorganic cation of plant cells, being essential for several basic physiological functions such as protein synthesis and enzyme activation, and must actively be taken up by ion transporters (Rodriguez-Navarro 2000). Excess external Na^+ can, however, impair K^+ acquisition and accumulation of Na^+ results in a low K^+ : Na^+ ratio that impairs cellular functioning (Pardo and Quintero 2002). We noted that inoculated plants in our study had significantly lower K^+ concentrations than uninoculated plants, which could have also influenced disease severity. The prevailing view is that a high K^+ status decreases the incidence of many diseases. The beneficial effect of K^+ is most obvious for fungal and bacterial diseases where up to 70% of studies report a decrease of disease incidence (Amtmann *et al*. 2008). Our study showed that the diseased plants had reduced K^+ concentrations, suggesting in some way that the disease (pathogen) had reduced K^+ concentration, slowing down enzyme activity and further influencing cellular functions.

Saline soil poses both water deficit and ion toxicity challenges that can have complex consequences for both the pathogen and the host plant (Snapp *et al*. 1991). Osmotic stress, ion imbalance in cells (especially lower concentrations of K^+), and Na^+ and/or Cl^- toxicity (Tavakkoli *et al*. 2011) could all weaken plants, as well as encouraging growth of pathogens, resulting in more severe disease occurrence. Our study showed that the inoculated common bean had significantly higher tissue Na^+ and lower K^+ concentrations than uninoculated plants. This demonstrates the importance of maintaining the appropriate balance between K^+ and Na^+ in areas where *M. phaseolina* is a serious pathogen. Further, our study showed hypocotyls from inoculated plants contained less K^+ and more Na^+ than shoots and that pathogen spread within hypocotyl tissues was rapid following hypocotyl inoculation.

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