International Journal of Agronomy

Cultivated Legume Species

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Editorial Board

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Editorial
Cultivated Legume Species

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Legume species belong to the Fabaceae family and are characterized by their fruit, called usually pod. Members of the legume family, the Fabaceae or Leguminosae, fill critical niches in most terrestrial biomes. The family has traditionally been divided into three subfamilies: Caesalpinioideae, Mimosoideae, and Papilionoideae; this latter subfamily contains most of the major cultivated food and feed. Several legume species were domesticated by man, such as soybean, common bean, pea, faba bean, chickpea, lentil, peanut, pigeon pea, and cowpea. Some of these species are the basis for direct human nutrition, and animal feed. Immature pods or dry seeds of legumes are consumed, which have high protein content. Globally, grain legumes are the most relevant source of plant protein for human food and animal feed, but there are some constraints in their production, such as a poor adaptation, pest and diseases, and unstable yield.

Overweight or obesity, diabetes, metabolic syndrome, and cardiovascular diseases are exponentially growing human health issues. Nutritional and dietary actions are effective through a global shift in diet and reduced intake of energy-dense foods. Legume seeds represent one of the most promising foods to match these needs. This is due to their chemical composition and unique biological properties. Nonetheless, the nutritional value of legume seeds may be limited by the presence of antinutrient compounds whose reduction is desirable. Therefore, research activities aiming at exploring the possible formulation of grain legumes into easy-to-cook, ready-to-eat healthy food commodities are essential. To this purpose, the selection and optimal knowledge of the starting materials and the specific roles played by single seed components or fractions are crucial to envisage and design innovative food applications or to improve the traditional methods.

Legumes contribute to a sustainable improvement of environment when grown in mixture or rotations with other crops due to their ability to fix atmospheric molecular nitrogen in symbiotic association with rhizobia, and their effects on soil, control of weeds, and to serve as pollinators and cover crops. Legumes play a key role in the diversification and sustainable intensification of agriculture, particularly during climate change, sustainability of the food and feed chain, and meeting requirements of citizens for safe, healthy, and affordable food by preventing diet-related diseases and depression.

Crop production systems involving legumes represent a cheaper and more sustainable alternative to conventional practices by biological nitrogen fixation, thus reducing the use of industrially produced nitrogen fertilizers to improve yield of field crops. Improvement of N management is needed not only to optimize economic returns to farmers but also to minimize environmental concerns associated with the use of chemical fertilizers, namely, leaching problems and water pollution. Intercropping or crop rotation including legumes is a promising strategy for more sustainable crop production in many agricultural systems. For example, in a crop rotation, cultivated legumes can be used in between the cereals or other crops (e.g., vegetables) and have the potential of reducing biotic stresses, improving nutrient use efficiency, and contributing to increase yield and yield stability.

Currently global research projects involving genomic and genetic approaches to reach a deeper knowledge of economically important legume species are in progress, aimed to the genetic improvement in the near future.

This special issue includes six research articles related to the above mentioned topics.

"*Changes in protein, nonnutritional factors, and antioxidant capacity during germination of L. campestris seeds*" (C. Jiménez Martínez et al.) discusses that concentration of phenolic compounds increased 450% during germination from the original level in the seed. Thus, germination could be used to produce legume foods for human consumption with better nutraceutical properties.

"*Effect of soil pH on the alkaloid content of Lupinus angustifolius*" (G. Jansen et al.) presented results that clearly showed that the alkaloid content was significantly influenced by the soil pH. Also, genotypic differences in response to different pH values in the soil were observed.

"*Growth and physiological responses of Phaseolus species to salinity stress*" (J. S. Bayuelo-Jiménez et al.) shows that despite some changes in soluble carbohydrate accumulation induced by salt stress being detected, no consistent association with osmotic adjustment could be found in this study. Therefore, the authors suggest that tolerance to salt stress is largely unrelated to carbohydrate accumulation in *Phaseolus* species.

"*Genetic transformation of common bean (Phaseolus vulgaris L.) with the Gus color marker, the Bar herbicide resistance, and the barley (Hordeum vulgare) HVA1 drought tolerance genes*" (K. Kwapata et al.) discusses that some common bean varieties were genetically transformed via the biolistic bombardment of the apical shoot meristem primordia. Transgenes included *Gus* color marker which visually confirmed transgenic events, the *Bar* herbicide resistance selectable marker used for in vitro selection of transformed cells and which confirmed Liberty herbicide resistant plants, and the barley (*Hordeum vulgare*) late embryogenesis abundant protein (*HVA1*) which conferred drought tolerance with a corresponding increase in root length of transgenic plants.

"*Role of pigeonpea cultivation on soil fertility and farming system sustainability in Ghana*" (S. Adjei-Nsiah) shows that despite its importance, the potential of pigeonpea as a soil fertility improvement crop has not been exploited to any appreciable extent and the amount of land cultivated to pigeonpea in Ghana is very negligible. One conclusion of this study is that in order to promote the cultivation of pigeonpea in Ghana, there is the need to introduce varieties that combine early maturity with high yields and other desirable traits based on farmers preferences.

"*Quantitative trait loci analysis of folate content in dry beans, Phaseolus vulgaris L.*" (S. Khanal et al.) shows that folates are essential vitamins and folate deficiencies may lead to a number of health problems. Among the different forms of folates, 5-methylterahydrofolate (5MTHF) comprises more than 80% of the total folate in dry beans. A single marker QTL analysis identified three QTL for total folate and 5MTHF contents in the first measurement and one marker for the total folate in the second measurement in the F_2. These QTLs had significant dominance effects and individually accounted for 7.7 to 10.5% of total phenotypic variance. The total phenotypic variance explained by the four QTL was 18% for 5MTHF and 19% for total folate in the first measurement, but only 8% for total folate in the second measurement.

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Research Article

Quantitative Trait Loci Analysis of Folate Content in Dry Beans, *Phaseolus vulgaris* L.

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Dry beans (*Phaseolus vulgaris* L.) contain high levels of folates, yet the level of folate may vary among different genotypes. Folates are essential vitamins and folate deficiencies may lead to a number of health problems. Among the different forms of folates, 5-methyltetrahydrofolate (5MTHF) comprises more than 80% of the total folate in dry beans. The objectives of this paper were to compare selected genotypes of dry beans for the folate content of the dry seeds and to identify quantitative trait loci (QTL) associated with the folate content in a population derived from an inter-gene-pool cross of dry beans. The folate content was examined in three large-seeded (AC Elk, Redhawk, and Taylor) and one medium-seeded (Othello) dry bean genotypes, their six F_1 (i.e., one-way diallel crosses), and the F_2 of Othello/Redhawk that were evaluated in the field in 2009. Total folate and 5MTHF contents were measured twice with one-hour time interval. The significant variation ($P < 0.05$) in the folate content was observed among the parental genotypes, their F_1 progeny, and members of the F_2 population, ranging from 147 to 345 μg/100 g. There was a reduction in the 5MTHF and total folate contents in the second compared to the first measurement. Dark red kidney variety Redhawk consistently had the highest and pinto Othello had the lowest total folate and 5MTHF contents in both measurements. A single marker QTL analysis identified three QTL for total folate and 5MTHF contents in the first measurement and one marker for the total folate in the second measurement in the F_2. These QTL had significant dominance effects and individually accounted for 7.7% to 10.5% of the total phenotypic variance. The total phenotypic variance explained by the four QTL was 18% for 5MTHF and 19% for total folate in the first measurement, but only 8% for total folate in the second measurement.

1. Introduction

Dry beans (*Phaseolus vulgaris* L.), in addition to being excellent sources of protein and dietary fibre [1], are also good sources of minerals such as iron, calcium, and zinc, as well as folate [2]. Folate is the general term used to refer to different chemical forms of vitamin B_9 [3]. Naturally occurring forms of folate include tetrahydrofolate, 5-methyl tetrahydrofolate (5MTHF), 5-formyltetrahydrofolate, and 10-formyltetrahydrofolate [3]. Among the naturally occurring forms, 5MTHF is the most dominant and readily available form found in plant and animal metabolic cycles [4]. Previous research has indicated that 5MTHF comprises more than 85% of total folate in some major vegetables and close to 100% in some fruits [5]. Hence, in most studies, 5MTHF has been chosen as an indicator for the measurement of total folate present in dry beans. Moreover, 5MTHF is stable in acidic environments, which makes it possible to extract and analyse it with accuracy using High Pressure Liquid Chromatography (HPLC) [6].

Folates are required in human diets, because humans lack enzymes to synthesize folate *de novo* [7]. Folate deficiency in humans leads to a number of serious diseases. The occurrence of neural tube defects in infants and different forms of dementia and cardiovascular diseases in adults are due to the deficiency of folate [8]. The current recommended daily dietary allowance of folate is 400 μg for adults and 600 μg for pregnant women and lactating mothers. Folate-rich diets

are typically suggested for women planning a pregnancy or who are already pregnant, because of the essential role that folate plays in the production of nucleotides and many other metabolic processes during cell division [8].

Dry beans generally differ in many traits including seed composition and mineral concentration [9]. Despite high levels of folate in dry beans, limited data may also point to possible differences in the folate content of the genotypes from the two common bean gene pools. This variation may suggest the possibility of improving the folate content of dry beans through plant breeding. The objectives of this paper were, therefore, to (1) compare four varieties of dry beans from the two gene pools, their six F_1, and F_2 of Othello/Redhawk for the folate content in seeds and (2) identify quantitative trait loci (QTL) associated with the folate content in the F_2 of Othello/Redhawk.

2. Materials and Methods

2.1. Plant Materials and Field Trials. Three large-seeded varieties, SVM Taylor Horticulture cranberry bean, henceforth referred to as Taylor, AC Elk light red kidney bean, and Redhawk dark red kidney bean, were selected from the Andean gene pool and the medium-seeded variety, Othello pinto bean, was selected from the Middle America gene-pool. A one-way diallel cross between four parents was made in a growth room at the University of Guelph in the summer of 2009. All six F_1 hybrids were grown in the growth room and allowed to produce F_2 seeds.

The four dry bean varieties, six F_1, and six F_2 were grown in the field at the University of Guelph, Elora Research Station, near Elora, ON in the summer of 2009, in a randomized complete block design with three replications. After the harvest and folate measurement, only the F_2 of Othello/Redhawk was chosen for further analysis (see below). Each experimental plot of the four varieties and six F_1 consisted of a single row, 1.5 meter long with 0.76 meter row spacing. A maximum of 80 seeds of the F_2 of Othello/Redhawk were distributed uniformly in six rows, 1.5 meter long with 0.76 meter row spacing. The distance between each plant in the rows was 0.15 meter. At maturity, three plants from each plot of the four varieties, single plants from F_1, and every single plant from the F_2 of Othello/Redhawk were hand harvested in paper bags and were kept in a dryer at 30°C for 48 hours. Plants were then stored at room temperature until threshed. A single plant thresher was used to thresh one plant at a time. Seeds were collected in separate paper bags. Seeds were cleaned and seed moisture was measured using an Automatic Moisture Meter (Motomco 919E, Paterson, NJ, USA). Dry seeds were stored at −30°C until the folate was extracted.

2.2. Folate Measurement. The folate content was first measured only for four varieties and their six F_1. After the data was analyzed, the F_2 of Othello/Redhawk was chosen for the genetic analysis, from which every F_2 plant was analyzed. The folate content was measured using HPLC with fluorescence detection method at Agriculture and Agri-Food Canada, Guelph Food Research Center (GFRC), Guelph, ON. For each sample, two measurements were taken, with one hour time-interval. Extraction, enzymatic treatment, purification, preparation of standards and HPLC were carried out following the methods explained by Xue et al. [10].

2.3. Statistical Analyses. Raw data for total folate and 5MTHF in the first and second injections were first compiled and analyzed for four varieties and their six F_1. The raw data was subjected to analysis of variance using the PROC GLM procedure of SAS v9.2 [11], with folate content as the dependent variable and replication and genotype (varieties and F_1s) as independent variables. Sums of squares of genotype were partitioned into sums of squares of varieties, crosses, and parents versus crosses. Least square mean values and their standard errors were computed using the LSMEANS statement in PROC GLM. The F_2 of Othello/Redhawk was chosen for the genetic study because of highest significant differences between the two varieties (Table 1).

Statistical analyses of the folate content of the F_2 of Othello/Redhawk were conducted using the PROC MIXED procedure in SAS 9.2 using the codes provided by Scott and Milliken [12] for the Modified Augmented Randomized Complete Block Design, in which varieties and F_1 hybrids were the repeated checks and each F_2 individual was one experimental unit. To construct the model, two new variables were defined in the data set. The variable C takes the value of a check's name for checks (varieties and F_1s) and the value 0 for experimental units (each F_2 single plant). The following model was used:

$$Y_{ij} = \mu + r_j + c_i + x_i\left(c_i\right) + \varepsilon_{ij}, \quad (1)$$

where μ is the population mean, r_j denote the replication effect, and c_i and $x_i(c_i)$ denote the entry effect, that is, checks and the F_2 individuals. Replications were considered random and checks were considered fixed effects. The nested effect of entry (check) was considered random in the first run of the analysis to generate the Best Linear Unbiased Predictors (BLUP) for each one of the F_2 individuals using SOLUTIONS statement in PROC MIXED in SAS [11] and then considered fixed in a second run of the analysis to generate least square means values for the F_2 individuals. The least square means and BLUP values were linearly correlated with a coefficient of correlation greater than 0.99 and, therefore, only least square mean values were used for the QTL analyses.

2.4. DNA Extraction. Leaf tissue samples were taken from young trifoliolate of the four varieties, six F_1, and F_2 of Othello/Redhawk single plants when the first trifoliolates were fully expanded. Samples were stored on ice in a cooler until transferred to a −80°C freezer. DNA was extracted using a modified FastPrep (Sigma) extraction method following the manufacturer's protocol. Approximately, one cm^2 of frozen leaf, excluding the mid-rib, was placed into a screw-cap tube between a small and a large grinding bead with 600 μL of extraction buffer (200 mM Tris pH 7, 250 mM NaCl, 250 mM EDTA pH 8.0, 0.5% SDS, H_2O). The samples were homogenized in a FastPrep (Sigma) grinding machine (Thermo Electron Corporation, Milford, MA, USA) for 20 seconds at 4°C and placed on ice for 5 minutes. The homogenate was

Table 1: Least squared means, standard errors, and the summary of ANOVA for four measurements of total and 5-methyl tetrahydrofolate contents of four dry bean varieties, their six F_1, and the F_2 of Othello/Redhawk measured in two injected solutions at one-hour interval.

	First injected solution (μg/100 g)		Second injected solution (μg/100 g)	
	5-Methyltetrahydrofolate	Total folate	5-Methyltetrahydrofolate	Total folate
Parents				
Taylor	220.8	255.1	168.9	191.1
Othello	199.1	217.2	147.4	167.2
Redhawk	244.4	345.4	232.6	321.0
AC Elk	231.6	338.0	194.3	303.0
F_1 Crosses[1]				
Taylor/Othello	237.3	306.2	193.0	275.8
Taylor/Redhawk	282.5	355.5	233.7	314.8
Taylor/AC Elk	213.0	275.7	185.5	245.1
Othello/Redhawk	219.1	299.3	192.3	265.8
Othello/AC Elk	233.0	306.4	212.3	279.0
Redhawk/AC Elk	253.3	311.4	167.7	266.1
SE	29.00	31.89	23.90	26.28
F_2 of Othello/Redhawk				
Mean	218.0	236.8	181.1	195.2
Minimum	205.5	224.4	172.4	185.6
Maximum	230.5	249.3	189.8	204.7
SE	6.37	6.35	4.44	4.88
ANOVA				
Genotype	ns	ns	*	**
Parents	ns	*	**	**
Crosses	ns	ns	ns	ns
Parents versus crosses	ns	ns	ns	ns

* and ** are significant at $P < 0.05$ and 0.01, respectively; ns is not significant.
[1]In each cross, first parent is the female and second parent is the male.

pipetted into a new 1.5 mL tube and centrifuged for 5 min at 13200 rpm. An aliquot (400 μL) of the supernatant was transferred to a new 1.5 mL tube and 400 μL of cold isopropanol was added. The tubes were left at room temperature for 5 min and centrifuged for 5 minutes at 13200 rpm. All the supernatants were discarded and the DNA pellets were drained by inversion for 15 minutes and vacuum dried for 10 minutes. Doubled distilled water (500 μL) was added to the samples and was placed at 4°C overnight. The following day, the samples were centrifuged for 1 minute at 13200 rpm. A 480 μL aliquot of the supernatant was collected in a new tube and stored at −20°C until used.

2.5. Molecular Marker Genotyping. The parental lines and the F_2 population were genotyped with SNP markers developed by Shi et al. [13]. Genotyping was performed at the Genome Quebec Innovation Center (Montreal, QC) using the Sequenom iPLEX Gold Assay (Sequenom, Cambridge, MA).

2.6. Linkage Mapping. Linkage maps with 67 polymorphic SNP loci were constructed using the software Joinmap 4.0 [14]. Each marker locus was tested for conformity to the expected genotypic ratio of 1 : 2 : 1 in an F_2 population using the χ^2 test in Joinmap 4.0. The markers were first ordered into linkage groups using the "group" command (parameter value LOD > 5). The remaining markers were added to the respective linkage groups based on previous mapping information [14] using the "assign" command. An alignment comparison of the linkage map was done using the linkage map of McConnell et al. [15] as a reference map.

2.7. QTL Analysis. A single factor QTL analysis, using one-way ANOVA was used to find the association between polymorphic markers and folate content parameters using PROC GLM in SAS v9.2 [11] with the significant threshold of $\alpha = 0.05$. The best fit linear model was calculated for each marker and to R_p^2 of the model was computed to estimate the proportion of phenotypic variation explained by each marker. The mathematical model for the single marker QTL analysis was

$$y_i = \mu + x_i + \varepsilon_i, \quad (2)$$

where y_i is the phenotypic value, μ is the overall mean, x_i is the genotype score of the ith marker, and ε_i is the residual

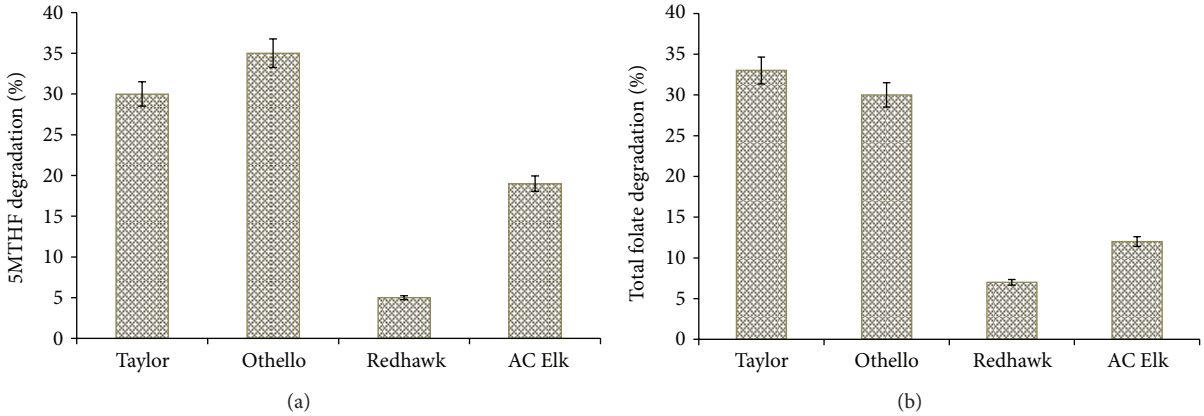

Figure 1: Instability of 5-methyltetrahydrofolate (5MTHF) (a) and total folate (b) contents estimated as the proportion of folate content lost in the one-hour time interval between two High Pressure Liquid Chromatography measurements in four dry bean varieties.

error. Forward stepwise regression was conducted for significant markers for each trait from single factor QTL analysis to estimate the total phenotypic variance explained by all QTL in the model. The significance of the additive effect at each SNP locus was tested using a single degree of freedom test using ESTIMATE statement in the PROC GLM procedure, in which the phenotypic value of the two homozygous genotypic groups were compared. Similarly, the significance of the dominance effect at each SNP locus was tested with a single degree of freedom contrasts, in which the phenotypic value of the heterozygous genotypic group was compared with the average phenotypic value of the two homozygous genotypic groups. The additive effect at each significant locus was then estimated as half of the difference of the two homozygous genotypic groups and the dominance effect was estimated as the difference of the phenotypic value of the heterozygous group and the mid value of the two homozygous groups.

3. Results

3.1. Folate Content of Parental Lines and Their F_1 Hybrids. Total folate and 5MTHF contents in the solution injected after one hour were significantly different ($P < 0.05$) among genotypes. The four varieties were significantly different ($P < 0.05$) for all four parameters except 5MTHF in the first injected solution (Table 1). Total folate content ranged between 217 and 345 μg/100 g in the first injected solutions and 167 and 321 μg/100 g in the second injected solutions (Table 1). The 5MTHF comprised 70% to 91% of total folate contents in the first injected solution and 72% to 88% of the total folate contents in the second injected solution. Folate contents of Taylor and Othello were significantly lower than AC Elk and Redhawk (Table 1). The reduction of 5MTHF content and total folate content in one-hour time interval between the first and second injections, used as a measure of folate instability, was highly variable for all four parental lines ranging from 5% to 30% for 5MTHF and 7% to 33% for total folate. Othello and Taylor had higher rates of instability of total folate content than Redhawk and AC Elk (Figure 1).

In the analysis of variance, the effect of parent versus crosses was not significant. However, the least square means of the Taylor/Redhawk for all four parameters deviated from mid-parent value towards the high folate content parent (Redhawk) pointing to the involvement of dominance and/or overdominance gene effects in that cross. Similarly, for Taylor/Othello the least square mean values of the F_1 for 5MTHF and total folate contents in the first injected solution deviated towards the high folate content parent (Taylor). On the other hand, the 5MTHF and total folate contents for second injected solution of the F_1 of Redhawk/AC Elk deviated towards the low folate content parent AC Elk (Table 1).

3.2. Folate Content of the F_2 of Othello/Redhawk. The frequency distribution of the folate content for the F_2 of Othello/Redhawk was continuous for all four parameters with the presence of transgressive segregants at both ends of the frequency distribution. While the population means for all four parameters were between the values of two varieties, the F_1 values were significantly higher than the population means for total folate (both measurements) and were shifted towards the high folate content parent, Redhawk (Figure 2).

The average loss of total folate content in one-hour time intervals from the first injected solution to the second injected solution was 30% for Othello (low folate content parent), but only 7% for Redhawk (high folate content parent). The average loss of folate in one-hour time interval from the first to the second injected solution was 17% for 5MTHF content and 18% for total folate content (Figure 2). Members of the F_2 population varied for the proportion of folate lost during the time interval between the first and second injected solutions as evidenced by low, but significant, coefficient of correlation between the folate contents in the first and second injected solutions. The correlations were $r = 0.46$ ($P < 0.001$) for total folate and $r = 0.30$ ($P < 0.001$) for 5MTHF content.

The 5MTHF comprised 70% to 90% of total folate content among the four varieties and F_2 individuals. There was highly significant positive correlation between 5MTHF and total folate in the first ($r = 0.87$; $P < 0.001$) and the second ($r = 0.70$; $P < 0.001$) injected solution.

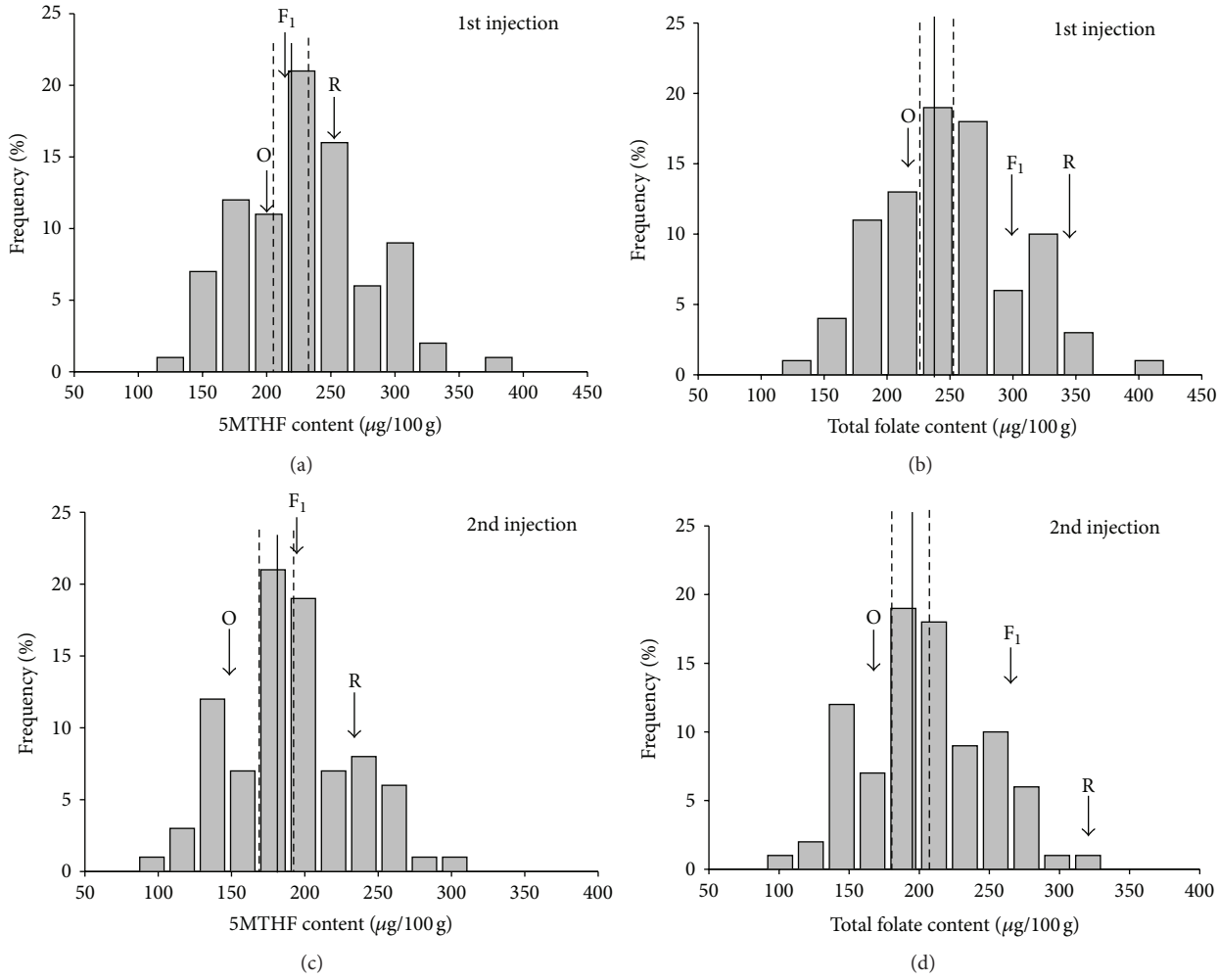

Figure 2: Relative frequency distribution of measurements of 5-methyltetrahydrofolate (5MTHF) and total folate content in the F_2 of Othello/Redhawk dry beans in the first ((a) and (b)) and second ((c) and (d)) injected solutions with one-hour time interval. Vertical lines indicate population mean and 95% confidence limits of the mean value in each plot, while arrows indicate the value for Redhawk (R), Othello (O), and their F_1.

3.3. SNP Analysis and Linkage Map. Among the tested SNP markers, 54% of them were polymorphic between the parents Othello and Redhawk. The total number of SNP markers included in the linkage map was 63, which resulted in a linkage map of 1056.14 cM, in 11 linkage groups (Figure 3), which is equivalent to the haploid chromosome number in the dry bean. The alignment of the linkage map with the map published by McConnell et al. [15] indicated that with the exception of a mismatch in Pv09 between markers g1286 and g544 all other markers were aligned. However, differences were observed in marker distances.

The chi-square test of conformity of the observed genotypic frequencies for the SNP markers with the expected 1 : 2 : 1 ratio indicated that 15 SNP markers out of a total of 63 markers had significant ($P < 0.05$) segregation distortion. Markers on Pv04 (g755), Pv05 (g1664 and g1883), Pv07 (g1065 and g2357), and Pv10 (g2521_B) were skewed towards Othello and markers on Pv11 (g1438 and g2135) were skewed towards Redhawk. While the segregation distortion of a section of Pv02 (g457 and g680_B) was observed towards Othello, marker g680 was skewed towards Redhawk. Similarly, the segregation distortion of a section of Pv08 (g1084) was observed towards Othello and markers g 2311and g1731 were skewed towards Redhawk.

3.4. Quantitative Trait Loci Analysis. One-way analysis of variance detected a total of four markers significantly ($P < 0.05$) associated with at least one of the folate content measurements (Table 2). These markers are located on Pv02 (g457_B), Pv09 (g1286, g2498), and Pv11 (g2135). Two markers, g1268 and g2498, on Pv09 explained 7.7% and 7.8% of the phenotypic variance, respectively, with a significant ($P < 0.01$) dominance effects for 5MTHF in the first injected solution. These markers (g1268 and g2498) were also significant ($P < 0.05$) for total folate in the first injected solution and explained 7.9% and 7.7% of the phenotypic variance, respectively, with a significant dominance effects. Neither of these markers, however, was significantly associated with

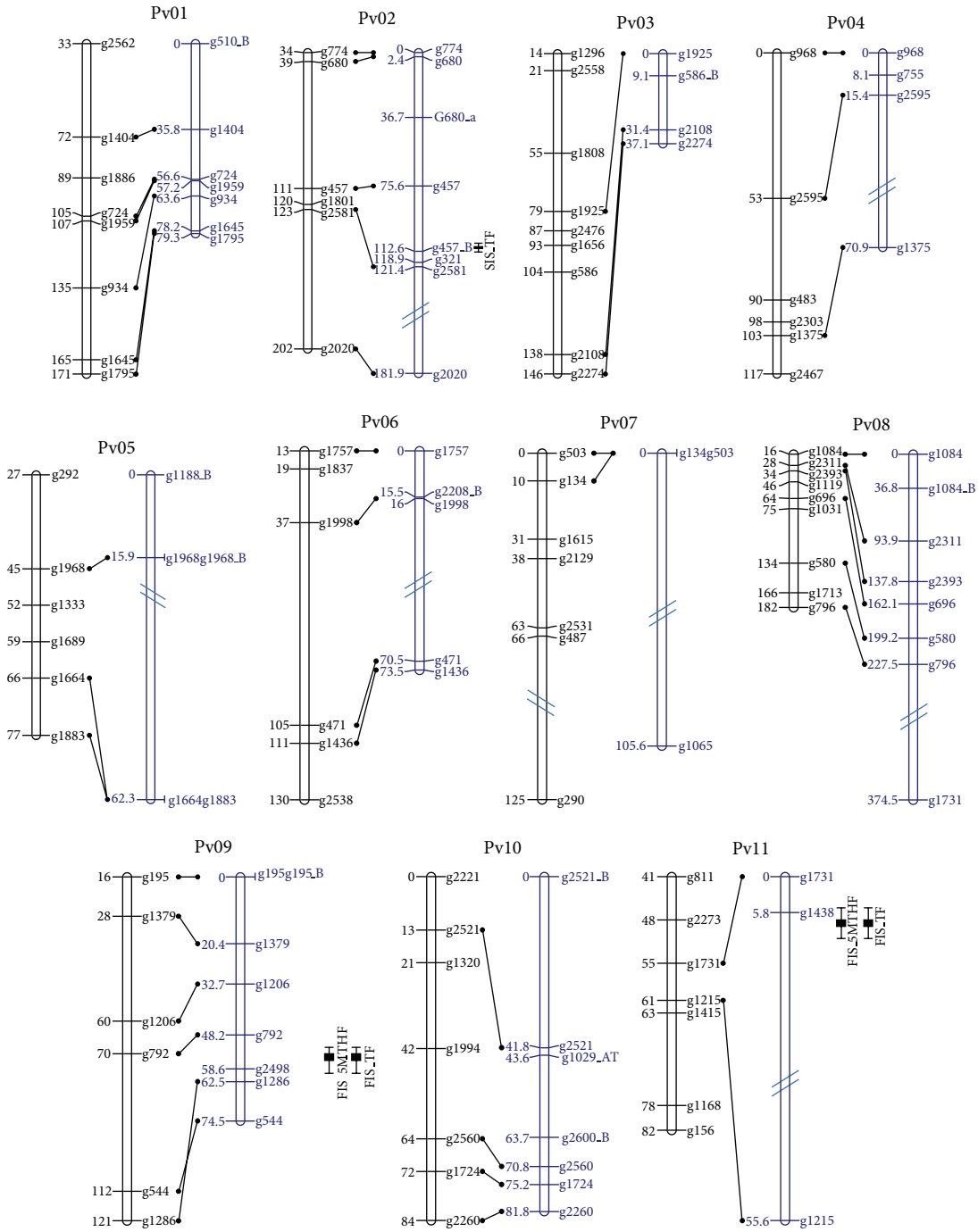

Figure 3: Alignment comparison of Othello × Redhawk single nucleotide polymorphic marker map (Linkage group on the left) with reference map (Linkage group on the right). The diagonal double-lines on the linkage groups represent the intervals with higher than 50 cM distance between pairs of adjacent markers. Approximate position of the QTL for total folate (FIS-TF) and 5-Methyltetrahydrofolate (FIS-5MTHF) in the first injected solution, and total folate in the second injected solution (SIS-TF) have been identified on the map. The hatched lines on the linkage groups identify the intervals with more than 50 cM genetic distance.

measurements taken at the second injected solution. The marker g457_B was the only marker with significant effect on total folate in the second injection, again with significant dominance effect, accounting for 8.1% of the total phenotypic variation. Marker g2135 on Pv11 was significant ($P < 0.01$) for measurements taken at the first injected solution with significant dominance effects, accounting for 9.3% and 10.5% of the variation. The total phenotypic variance explained by the significant markers in a multiple regression model was 18% for 5MTHF and 19% for total folate in the first injected solution, but only 8% for total folate in the second injected solution (Table 2).

Table 2: The single nucleotide polymorphism markers associated with the folate content in the F_2 of Othello/Redhawk in single marker quantitative trait loci analysis for the estimates of additive and dominance effects and the proportion of phenotypic variance accounted for by each locus.

			First injected solution								Second injected solution							
Marker	Position		5-Methyltetrahydrofolate				Total folate				5-Methyltetrahydrofolate				Total folate			
	Pv	cM	*P* value	Add.	Dom.	R_p^2	*P* value	Add.	Dom.	R_p^2	*P* value	Add.	Dom.	R_p^2	*P* value	Add.	Dom.	R_p^2
g457_B	2	112.6	ns	10.26	−2.37	1.2	ns	10.39	−1.43	1.1	ns	0.61	22.9**	4.8	0.04	0.89	25.39**	8.1
g1286	9	62.5	0.03	2.32	28.42**	7.7	0.03	2.71	31.08***	7.9	ns	3.65	−1.21	0.4	ns	4.22	−2.66	0.5
g2498	9	58.6	0.04	1.34	28.91**	7.8	0.04	1.15	30.98**	7.7	ns	0.97	−6.2	0.7	ns	0.07	−8.48	1.1
g2135	11	9.01	0.02	−11.24	24.37*	9.3	0.01	−13.14	27.45*	10.5	ns	−2.15	6.63	0.3	ns	−2.07	8.48	0.4
Total variance explained by significant markers						18				19				ns				8

Add: additive effect at each locus estimated as half the difference of the two homozygous genotypic groups.
Dom: dominance effect estimated as the deviation of heterozygous genotypic group from mid parental genotypes at each locus
R_p^2: the proportion of phenotypic variance accounted for by each locus.
* and ** are significant at $P < 0.05$ and 0.01; ns: not significant at $P < 0.05$.

4. Discussion

The results of this study confirmed that despite the high levels of the folate content in dry beans, the genetic variation still exists among different genotypes. Differences of up to 59% for 5MTHF and total folate contents in the first injected solution and up to 92% for 5MTHF content and total folate content in the second injected solution after one hour were observed. Results suggested that the four varieties were not only different for the level of the folate content, but also in its instability and that instability of folate in the extract may have contributed significantly in the variation observed here. Nevertheless, the variation in the folate content and stability in dry beans observed in this study was more pronounced among genotypes from different market classes. The large-seeded Redhawk, AC Elk, and Taylor had a higher folate content than the medium-seeded Othello. Islam et al. [9] also reported higher concentrations of minerals in the large-seed Andean beans compared with the small- and medium-seeded Middle American beans.

Continuous variation for the folate content, coupled with transgressive segregation, which was observed at both ends of the frequency distribution of folate contents in the F_2 of Othello/Redhawk, indicated that quantitative genetic factors are involved in the inheritance of the folate content in dry beans. Blair et al. [16] reported that the inheritance of iron and zinc accumulation in dry bean seeds was also predominantly quantitative in a recombinant inbred line population of dry beans derived from a cross of DOR364 (low)/G19833 (high). Cichy et al. [17], however, reported a monogenic inheritance for seed zinc accumulation.

The observation of F_2 individuals with folate contents higher than the high folate content parent or lower than the low folate content parent suggests that while the parental lines may share common genes for the folate content, they may have unique loci that interact in the segregating population to determine the folate content in the F_2 individuals. Moreover, low but significant correlations between folate content in the first and second measurements indicated that the F_2 individuals had a differential response to the one-hour time interval, which may suggest that breeding materials may be different in terms of stability of folate.

Significant marker-QTL associations were identified in this study. Four markers were associated with at least one of the four measurements of the folate content with three markers significantly associated with the folate content in the first injected solution and only one for the solution injected after one hour. The four markers were distributed among three linkage groups. Although most of these QTL did not have large effects individually, together these QTL could control a significant proportion of variation. Furthermore, only dominance effects were significant for these QTL. The small effects of the identified QTL for folate content, the presence of dominance gene effect, and the significant reduction of folate content in the second injected solution as a measure of folate instability, all point to difficulties associated with breeding for a high folate content in dry beans.

The major sources of folate in human diets are legumes, green leafy vegetables, wheat germ, egg yolk, livers, and fortified foods [3]. However, the bioavailability of folate depends on the processing methods and conditions of these sources. A previous study has indicated that large portions of the folate in edible beans are lost during canning processes [18]. A major loss of 5MTHF in navy beans was observed after soaking and cooking processes [10]. Johansson et al. [19] evaluated the folate content of 10 different precooked vegetarian ready-to-eat meals before and after reheating and reported a significant reduction in the folate content after reheating. In this study, a one-hour time interval between two injections in HPLC was used as a measure of the folate instability in the extract. Among the four varieties, Redhawk and AC Elk had the highest rate of stability in the one-hour time interval. While this may suggest that instability of folate may have contributed significantly in the variation reported here, it may also indicate the need for future research in this area.

Instability of folates in high temperature food processing and therefore folate degradation during cooking and canning processes [10, 18] and not the original level of folate content in dry beans appear to be the major limiting factor in the food produced from dry beans. Results presented here point to the presence of variation in the rate of instability in one hour time interval between the two HPLC injections. Other studies have reported the presence of proteins that bind to folate and protect folate during processing [20]. Protein binding also protects polyglutamyl folates from deglutamylation [21, 22]. Future research on folate content in dry beans should, therefore, be directed towards biochemical mechanisms that may protect the already high levels of folate in the dry beans.

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Research Article

Changes in Protein, Nonnutritional Factors, and Antioxidant Capacity during Germination of *L. campestris* Seeds

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The changes in SDS-PAGE proteins patterns, oligosaccharides and phenolic compounds of *L. campestris* seeds, were evaluated during nine germination days. SDS-PAGE pattern showed 12 bands in the original protein seeds, while in the samples after 1–9 germination days, the proteins located in the range of 28–49 and 49–80 kDa indicated an important reduction, and there was an increase in bands about 27 kDa. On the other hand, oligosaccharides showed more than 50% of decrease in its total concentration after 4 germination days; nevertheless after the fifth day, the oligosaccharides concentration increases and rises more than 30% of the original concentration. Phenolic compounds increased their concentration since the first germination day reaching until 450% more than the original seed level. The obtained results are related with liberation or increase of phenolic compounds with antioxidant properties, allowing us to suggest that the germination would be used to produce legume foods for human consumption with better nutraceutical properties.

1. Introduction

Legume seeds are important staple foods, particularly in developing countries, due to their relatively low cost, long conservation time, and high nutritional value; among these meals it is *Lupinus* seeds and their derivatives. This legume is one of the richest sources of vegetable protein, and although the protein content and amino acid profile vary between species, the intraspecies variability is low. In 2009, the FAOST reported that the area harvested was 662712 Ha, and *L. albus* and *L. angustifolius* were the most widely used. About 100 wild species have been reported throughout México [1]. These wild lupins have not been exploited at a commercial level. For this reason, in the present work we consider them as potential providers of vegetable proteins for human consumption. *Lupinus campestris* seed, like other *Lupinus* species, has high protein content (44%) [1, 2]. Lupin seeds offer some advantages in comparison with soy bean, since it contains only small amounts of trypsin inhibitors, tannins, phytates, saponins, α-galactosides, and so forth [3, 4]. However, a limitation for the wider use of lupins has been their high content of quinolizidine alkaloids [5, 6] as well as condensed tannins [7, 8]. Consequently, it is desirable to develop transformation processes which could improve the nutritional quality of legumes and also provide new derived products for the consumers. Germination is considered a potentially beneficial process for legume seed transformation which may decrease undesirable components such as alkaloids and phytates [9], and during germination, some grade of transformation of alkaloids to other more bioactive compounds, such as esters, occurs [7]. Cuadra et al. [3] and De Cortes-Sánchez et al. [7] found a slight increase in

alkaloids during germination of *L. albus, L. angustifolius*, and *L. campestris*, and no α-pyridone alkaloids, such as the highly toxic anagyrine and cytosine, were detected in any of these species. Germination also increases nutrients such as vitamin C [10] and increase protein digestibility [11], consequently improving nutritional quality. Additional advantages of germination are reduction in cooking time and improvement of the product sensorial attributes [11]. Germination has been shown to decrease the level of α-galactosides of different legume seeds including soybean, black bean, and lupin seed, with the corresponding decrease in carbohydrates available for fermentation in the large human intestine. The content of trypsin inhibitors and phytates is also decreased, but considerable amounts of these factors are still present after germination [6]. On the other hand, it is widely accepted that antioxidant activity of food is related to high phenolic content. Phenolic compounds are capable of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, and inhibiting oxidases [12]. Legume seeds are a rich source of many substances with antioxidant properties, including plant phenolics. *Lupinus* is a potential source of bioactive components with antioxidant activities. Although the interest in *Lupinus* species as a valuable component of functional food is increasing and has let to investigate on the determination of antioxidant activity in *Lupinus* seeds and its products, the information is scarce [13, 14]. The objective of this work was to evaluate the original content of proteins, oligosaccharides, and phenolic compounds, the antioxidant capacity in *Lupinus campestris* seed, and the changes of these parameters during the germination process.

2. Material and Methods

2.1. Samples and Germination Process. L. campestris seeds (wild type) were collected along 50 km of the Oaxtepec-Xochimilco highway in the Morelos State, México.

Germination process was performed as described by De Cortes-Sánchez et al. [7]. Briefly, 800 *Lupinus campestris* seeds were used for the germination assay distributed in 10 trays, with 80 seeds each one. The seeds were spread on a moist sheet of filter paper (Albet 1516, 42–52 cm) and covered with another sheet of moist filter paper. They were put into a germination chamber under environmentally controlled conditions: 20°C, 8 h of light per day exposure, and watering of the seeds during germination keeps the paper always wet. Samples (80 seeds/tray) were taken at 0 (control), 1, 2, 3, 4, 5, 6, 7, 8, and 9 germination days. The germination process was repeated twice, and the germination capacity was evaluated by germination percentage and seed weights. Samples for analysis were constituted by germinated and moist seeds, discarding those that did not show any water absorption during the process. The germinated seeds were freeze-dried, milled, and passed through a sieve of 0.5 mm. The germinated flour was stored in darkness in a desiccator at 4°C until analysis.

2.2. Gel Electrophoresis. Denaturing gel electrophoresis (SDS-PAGE) was carried out according to the method of Schagger and von Jagow [15] using 10% polyacrylamide gels in the presence of 1% SDS; the proteins (1 μg) were loaded with or without β-mercaptoethanol. Standards used were phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa).

2.3. Extraction and Quantification of Carbohydrates (CH). The method of Muzquiz et al. [16] was used for CH extraction. 0.1 g of grounded seeds was homogenized with aqueous ethanol solution (50% v/v, 5 mL) for 1 min at 4°C. Then the mixture was centrifuged for 5 min (2100 $\times$g) at 4°C, and the supernatant was recovered. The procedure was repeated twice, and the combined supernatants were concentrated under vacuum at 35°C. The concentrated supernatant was dissolved in deionized water (1 mL) and passed through a Waters minicolumn (Waters C-18 at 500 mg/cc) with Supelco vacuum system.

Samples (20 μL) were analyzed using a Beckman HPLC chromatograph f156 with refraction index detector. A Spherisob-5-NH_2 column (250 $\times$ 4.6 mm id) was used with acetonitrile: water (65 : 35, v/v) as the mobile phase at a flow rate of 1 mL min^{-1}. Individual sugars were quantified by comparison with standards of sucrose, raffinose, stachyose, and verbascose. Calibration curves were prepared for all these sugars, and a linear response was obtained for the range of 0–5 mg/mL with a determination coefficient (r^2) > 0.99.

2.4. Extraction and Quantification of Phenolic Compounds (PC). 1 g of sample was extracted with 10 mL methanol previous to phenolic determination. Total phenols content was estimated by using the Folin-Ciocalteu colorimetric method [17]. Briefly, the 0.02 mL of the extracts was oxidized with 0.1 mL of 0.5 N Folin-Ciocalteu reagent, and then the reaction was neutralized with 0.3 mL sodium carbonate solution (20%). The absorbance values were obtained by the resulting blue color measured at 760 nm with a Beckman spectrophotometer (California, USA) model DU-65 after incubation for 2 h at 25°C. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg of gallic acid equivalent per 1 g of dry weight.

2.5. TLC Analysis of Phenolic Compounds. TLC was performed on TLC sheets coated with 0.25 mm layers of silica gel 60 F254 (E. Merck, number 5554). Two mobile phases were used: ethyl acetate-formic acid-ethanol (65 : 15 : 20, v/v/v) and 1-butanol-acetic acid-water (7 : 0.5 : 2.5, v/v/v), upper phase. The chromatograms were evaluated in UV light at 360 nm before spraying them with 10% sulphuric acid [18].

2.6. HPLC Analysis of Phenolic Compounds. HPLC analysis was performed on an Agilent Technologies 1200 series liquid chromatograph (G1311A quaternary pump, UV-VIS DAD G1315D detector, ALS G1329A injector, G1322A Deggaser, and TCC G1316A thermostat column), equipped with a Zorbax Eclipse XDB-C18 column (150 $\times$ 4.6 mm, 5 mm particle size) (Agilent Technologies, USA), and thermostated at 30°C. A gradient elution was used to separate the extracted phenolics. Solvent (A) was 5.0% formic acid in water, and

solvent (B) was acetonitrile. Elution was performed at a solvent flow rate of 1.0 mL/min. The gradient profile of the system was 0% solvent B at the initial stage, 0% solvent B at 3 min, 30% solvent B at 5 min, 60% solvent B at 20 min, 100% solvent B at 25 min, and 0% solvent B at 30 to 35 min.

The eluted phenolic compounds were monitored at 280 nm. Quantitative levels were determined by comparing with a catechin standard curve. Phenolic concentration was expressed as mg catechin equivalent per gram of dry sample.

2.7. Free Radical DPPH Scavenging Capacity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical used for assessing antioxidant activity. Reduction of DPPH by an antioxidant or by a radical species results in a loss of absorbance at 515 nm. PC extracts were adjusted at a concentration of 0.24 mg gallic acid equivalent/mL prior to antioxidant capacity evaluation. Determination of antioxidant capacity, previously adapted for microplates [19], was performed as follows: 0.02 mL of extract (500 μM gallic acid equivalent) or standard (gallic acid, 500 μM) was added to a 96-well flat-bottom plates containing 0.2 mL of DPPH solution (125 μM DPPH in 80% methanol). Samples were prepared in triplicate. The plate was covered, left in the dark at room temperature, and read after 90 min in a visible-UV microplate reader (680 XR Microplate Reader, Bio-Rad Laboratories, Inc) using a 520 nm filter. Data are expressed as a percentage of DPPH· discoloration [20].

2.8. Statistical Analysis. All analyses were carried out in triplicate, and the report data are the average of the results and the standard error in each case.

3. Results

3.1. Germination. In Figure 1 it is shown the germination capacity expressed as percent of germinated seeds. This germination percentage increases from day 1 to day 4, and after that, no significant increase in germination is observed.

The gain in weight is observed in Figure 1, an increase in weight can be observed since the first day, this weight augmentation was due mainly to the water that has shrunk, and the germination percentage was 5% only. The total increase in weight was three times plus from the initial weight of the seed. By the second day, the germination and the weight have increased to 27% and 14 g/80 seeds (Figure 1), and additionally root has left the head. The greatest increment in the number of germinated seeds (from 27% to 82%) is observed between the second and third day. At the fourth day, 98% of the seeds showed development of the stem and root. A maximum germination percentage of 100% was obtained, which shows the good viability of *L. campestris*. These results agree with De Cuadra et al. [3] who reported a high degree of germination (up to 100%) for two *Lupinus* species.

3.2. Electrophoretic Analysis. Figure 2 shows the electrophoretic profile of *L. campestris* seeds subjected to different germination times. As it is observed the seed without any germination time showed greater amount of protein bands located between 20 and 75 kDa. As the germination time advances, the proteins located in the range of 28–49 and 49–75 kDa almost disappeared after nine germination days of Lupinus seed, and there was an increase in bands about 27 kDa. These results confirm previous findings about storage proteins, which are hydrolysed and mobilised after germination [21, 22]. This behavior lets us to suggest that the principal storage protein molecules, the globulins 7 s, and 11 s constituted by three and six subunits, respectively, were hydrolyzed in lower molecular weight compounds which has a best digestibility and consequently a better biological value.

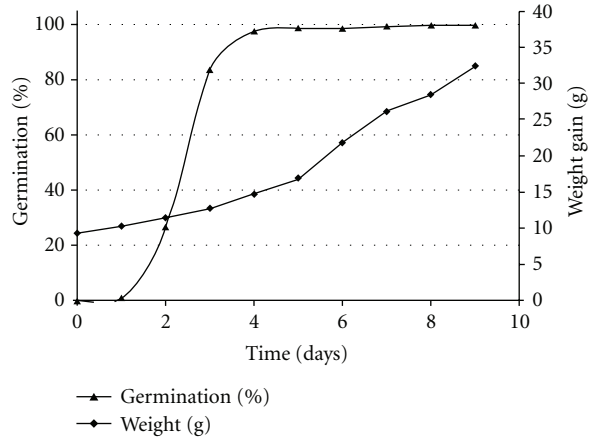

Figure 1: Germination (%) and weight gain (g) of the *L. campestris* seeds. The results represent the average of three independent experiments ± S. E.

3.3. Changes of Oligosaccharides in L. campestris Seeds during the Germination. The *L. campestris* germinated seeds were also evaluated as for the variation of present oligosaccharides. The obtained results are showed in Table 1. The concentration of total oligosaccharides in the seed without germinating was of 90.26 mg/g; this concentration was diminished near 15% in the first day, and then 25, 46, and 58% in the period were comprised since the second to the fourth germination day. Then oligosaccharide concentration increased its value from the fifth to the ninth day, reaching 30% above than the original content. The composition of oligosaccharides varies during the germination process. In the seed without treatment, the sucrose was present with an initial content of 21.45 mg/g increasing to 55.36 mg/g at five days of germination. Since the sixth day, sucrose diminished until reaching 14.84 mg/g of seed at the nine day of germination. This increase in sucrose concentration can be due to hydrolysis of oligosaccharides by the α-galactosidase enzyme, which selectively acts on the galactosides such as raffinose, stachyose, and verbascose releasing sucrose [23]. Muzquiz et al. [16] has reported a similar behavior in other species of *Lupinus*. After the fourth day of germination, the oligosaccharides proportion has increased substantially, mainly in the stachyose percentage, which is almost twice of the originally presented. Even though there is a substantial reduction of these carbohydrates, they are not totally eliminated, since it has been informed for other species of *Lupinus* whose diminution is bigger than 80–100% after four days

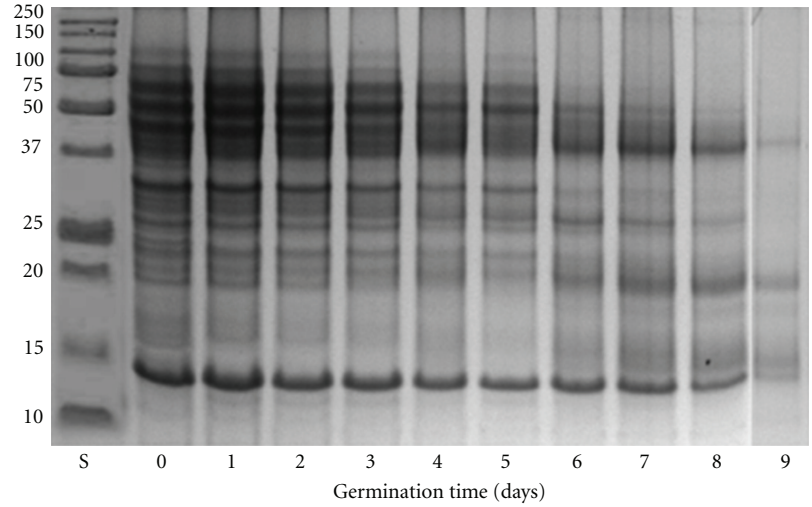

Figure 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of present proteins in the *L. campestris* germinated seed. S = standards (kDa).

Table 1: Behavior of carbohydrates of the *L. campestris* germinated seed (mg/g of seed).

Time (days)	Sucrose	Raffinose	Stachyose	Verbascose	Total oligosaccharides
0	21.45 ± 0.76	13.65 ± 0.89	57.16 ± 0.95	19.45 ± 0.59	90.26 ± 0.73
1	26.49 ± 3.86	8.51 ± 0.51	54.35 ± 3.38	11.48 ± 2.14	74.34 ± 1.17
2	32.21 ± 1.80	7.92 ± 0.51	49.10 ± 0.51	10.94 ± 0.55	67.96 ± 0.02
3	34.38 ± 1.37	6.49 ± 0.68	36.75 ± 1.06	5.53 ± 1.06	48.77 ± 0.03
4	46.14 ± 0.34	4.03 ± 1.03	29.99 ± 0.13	6.78 ± 0.12	40.80 ± 0.07
5	55.36 ± 5.15	7.08 ± 1.85	47.95 ± 0.69	8.44 ± 0.69	63.47 ± 0.65
6	35.09 ± 1.65	8.18 ± 2.23	65.17 ± 0.12	9.30 ± 0.12	82.65 ± 0.32
7	27.85 ± 1.45	10.19 ± 1.01	84.75 ± 0.57	9.64 ± 0.58	104.58 ± 0.13
8	22.95 ± 1.57	11.38 ± 0.10	93.09 ± 0.24	10.82 ± 0.24	115.29 ± 0.02
9	14.84 ± 3.76	11.34 ± 4.23	98.17 ± 2.53	11.93 ± 0.23	121.44 ± 0.30

*The values represent the average of two separated germinations with extractions made by triplicate ± S.E.

of germination [3, 24]. This difference can be due to the germination conditions in which the seeds were carried out. With the obtained results it is observed that the germination diminishes the concentration of oligosaccharides, being the lower value in the fourth day.

3.4. Phenolic Compounds in Seed of L. campestris during the Germination. Total phenolic compounds concentrations in the germinated *L. campestris* seed during nine germination days are presented in Figure 3. Control seed, without germination, presented 5.27 mg gallic acid equivalent per g of seed. This value remained almost constant during the days one and two. After that, the concentration of total phenolics increased gradually reaching twice the original value. However, phenolic content was in the range reported for other legumes such as yellow pea, green pea, lentils, common beans, and soybean [25] and similar to the content in other *Lupinus* species [26]. Contrary to the behavior of the oligosaccharides, the phenolic compounds increase as the time of germination occurs. The behavior shown for *Lupinus* germinated seeds differs from the observed by Muzquiz et al. [27] who indicated a reduction of 76% in phenolics from lentil but is similar to *Cajanus* seed [28] which showed a fivefold increment in total phenolic content during a period of five germination days.

3.5. Phenolic Compounds by HPLC. Although individual phenolics remain unidentified, they were quantified on the basis of a catechin calibration curve. In Table 2, the changes in composition and quantity of phenolics as determined by HPLC are shown.

There are two main groups of peaks. The first one which is presented since cero day increases at seven and eight days and decrease a little in the ninth day. This group is formed by peaks marked as peak 1 to 6 and peak number 10. The other group of peaks appears between the third and fourth day of germination, increases its concentration at the same days the other group does, and also diminished at nine day. This group is formed by peaks number 7–9 and 11–15 (Table 2). As the time of germination occurs, the complexity and quantity of total phenolics increase, being the germinated seeds on seven to nine day more complex in composition than no germinated seeds or those on the first germination days. Also, seeds in the seventh day are the richest in total phenolic composition.

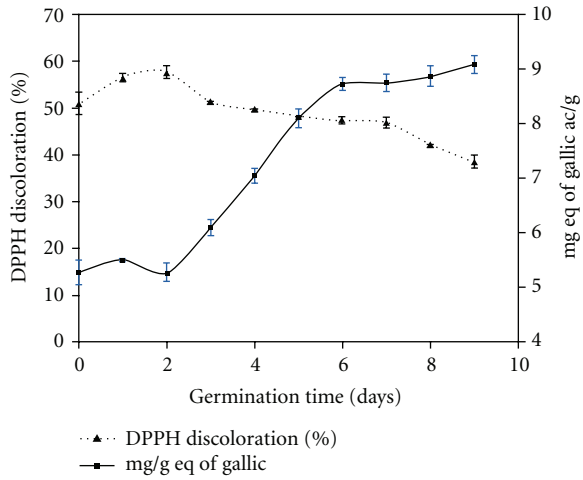

Figure 3: Phenolic compound variation and antioxidant capacity in *L. campestris* germinated seed.

Concentration of total phenolics by HPLC is lower than the obtained by the Folin-Ciocalteu method. Considering the heterogeneity of natural phenols and the possibility of interference from other readily oxidized substances such as ascorbic acid and mono- and disaccharides, this disagreement between methods is comprehensible [17, 29].

3.6. Antioxidant Capacity. All the extracts showed antioxidant capacity against DPPH-free radical, as measured by the decrease in absorbance at 520 nm. During the seeds germination, it was observed a light increase in antioxidant capacity nongeminated seeds until the second germination day (51–58%), followed by a continuing depression in antioxidant capacity until the ninth germination day (38%) (Figure 3). The initial antioxidant capacity (51%) is similar to that reported for other legumes [30]. An enhancement in antioxidant capacity by germination has been reported for *Lupinus albus* [14] and *Lupinus angustifolius* seeds [13] as measured in aqueous extracts. The *L. campestris* methanolic extracts showed a different behavior, which could be attributed to the kind of compounds that could be solubilized by methanol, and since water could solubilize other antioxidants such as vitamins. Fernandez-Orozco et al. [13] suggested that polyphenols extractability is better in buffer phosphate than in methanol. Correlations between antioxidant capacity toward DPPH-free radical and total polyphenols have been observed in beans [30] and in *L. angustifolius* germinated seeds [13]. In this study, polyphenol concentration did not correlate with antioxidant activity; while polyphenols increase as germination progress, antioxidant capacity decreases. It is interesting to note that polyphenol concentration was adjusted to 0.24 mg/mL in all samples, previously to antioxidant capacity determination. These results suggest again that it is composition but not concentration of polyphenols in the extracts, and possibly the presence of other antioxidants, which makes a difference in antioxidant capacity behavior. In order to confirm that composition affects antioxidant activity, the extracts were analyzed by TLC. The best profile was obtained with ethyl acetate-formic acid-ethanol (65 : 15 : 20, v/v/v), which is shown in Figures 4(a) and 4(b). 360 nm UV light shows that there is a spot with an Rf value of 0.375; although this yellowish fluorescent spot is in all samples, its relative intensity is bigger at the last germination days. There is another spot (Rf 0.875) that is present in all germination days (Figure 4(a)). On the other hand, the extracts would contain flavonoids and phenolic acids due to the yellow and the blue fluorescent bands under 360 nm UV light [18]. The Figure 4(b) shows the TLC plate revealed with sulphuric acid. There is a group of three spots at the medium of the plate in all extracts (Rf values = 0.424, 0.515, and 0.606); however it has higher intensity around five–seven days, this intensity suggests higher concentration of phenolics, as all the samples were applied in the same volume (Figure 4(b)). Another group of phenolics is observed in 0.031, 0.156, and 0.219 Rf values. The behavior of this second group differs from the previous one; the spots can be visualized in the seed without germination, and at one and two days, later the group is disappeared in the next two days, increased its intensity in fifth day, and once again, decreased in the last germination days. Changes in composition of the phenolic extract were confirmed by HPLC analysis, as described previously (Table 2) germination process increase, the complexity of the phenolic extract (Figure 4). There is a group of compounds around 25 min that should appear as a consequence of germination. According to the HPLC analysis these compounds must be lower polar, suggesting that their antioxidant activity could be less than more polar compounds.

The antioxidant activity of phenolic compounds is affected by their chemical structure. Structure-activity relationships have been used as a theoretical method for predicting antioxidant activity. Polymeric polyphenols are more potent antioxidants than simple monomeric phenolics: Hagerman et al. [31] demonstrated the higher antioxidant ability of condensed and hydrolyzable tannins at quenching peroxyl radicals over simple phenols; Yamaguchi et al. [32] observed that the higher the polymerization degree of flavanols, the stronger the superoxide-scavenging activity. A similar effect was reported for the capacity to inhibit the O_2^- radical, which increased with the degree of procyanidin polymerization [33].

The antioxidant activity also depends on the type and polarity of the extracting solvent, the isolation procedures, purity of active compounds, the test system, and substrate to be protected by the antioxidant [34].

4. Conclusion

The germination is a simple technological process of easy application and low cost. This process allows to the protein modification, obtaining peptides of low molecular weight and improving the nutritional quality. The oligosaccharides ones show diminution in the third germination day, nevertheless tend to increase as of the fourth day of this one process. On the contrary, the phenolic compounds concentration increases from the first day. With this, we can

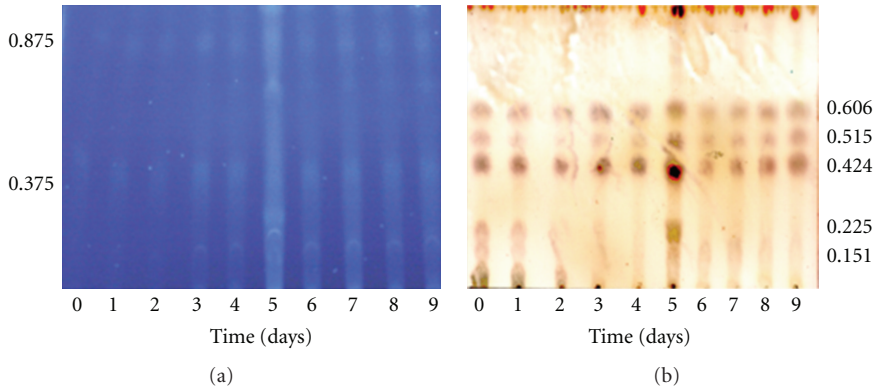

Figure 4: TLC analysis of phenolic compounds of *L. campestris* germinated seed eluted with a mixture of ethyl acetate-formic acid-ethanol (65 : 15 : 20, v/v/v) and revealed (a) UV light at 360 nm and (b) 10% sulphuric acid.

Table 2: Phenolic compounds in *L. campestris* germinated seeds by HPLC.

Peak number	Retention time (min)	Phenolic concentration, days of germination[1]									
		0	1	2	3	4	5	6	7	8	9
1	1.590	145.8	47.3	105.8	100.4	325.0	235.1	444.7	568.9	573.6	452.1
2	1.654	124.4	ND	76.4	118.4	238.9	252.1	347.0	499.2	498.0	408.9
3	1.764	362.8	ND	ND	ND	233.2	ND	231.0	306.8	310.7	272.5
4	7.454	524.7	133.6	149.7	108.3	321.6	137.2	357.7	474.2	482.8	431.3
5	8.941	665.0	ND	243.7	209.4	427.0	332.9	551.5	982.4	837.3	763.1
6	9.169	593.5	215.1	165.0	119.4	380.6	273.0	511.8	931.7	829.9	746.9
7	11.277	ND	ND	ND	ND	ND	111.4	ND	454.8	393.5	ND
8	12.038	ND	ND	ND	ND	ND	272.1	440.4	1270.5	1159.9	853.0
9	12.178	ND	ND	ND	ND	ND	162.2	257.3	696.4	785.6	561.1
10	12.626	395.7	64.5	98.8	ND	ND	134.7	288.5	451.1	400.7	341.3
11	14.455	ND	ND	ND	ND	ND	ND	214.6	354.0	308.4	265.1
12	15.953	ND	ND	ND	99.5	451.2	231.0	701.8	1157.9	668.6	452.8
13	25.195	ND	ND	ND	ND	212.4	87.0	318.9	262.2	253.2	209.4
14	25.361	ND	ND	ND	ND	85.4	ND	62.6	55.9	98.5	90.3
15	25.522	ND	ND	ND	ND	137.8	54.4	255.1	199.0	164.1	128.5
Total concentration		2811.9	460.5	839.5	755.4	2812.9	2282.9	4983.0	8665.1	7764.6	5976.3

[1] Phenolic concentration expressed as μg catechin equivalent per g of dry sample.
ND: not determined, under the detection limit.

conclude that it is necessary to control the time of germination to obtain an optimal concentration of nonnutritional factors to the third day.

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Research Article

Effect of the Soil pH on the Alkaloid Content of *Lupinus angustifolius*

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Field studies were conducted in growing seasons 2004, 2005, and 2010 to investigate the effect of different soil pH values on the alkaloid content in seeds of *Lupinus angustifolius*. Two-year experiments with eleven cultivars were carried out in acid soils with an average of pH = 5.8 (Mecklenburg-Western Pomerania) and on calcareous soils with an average pH of 7.1 (Bavaria), respectively. In addition, in 2010, eight cultivars were grown in field experiments in soils with pH values varying between pH = 5.3 and pH = 6.7. In all experiments conducted on soils with a higher pH (pH = 6.7 and pH = 7.1), a significantly lower alkaloid content was detected in all *Lupinus angustifolius* cultivars than on soils with a lower pH (pH = 5.3 and pH = 5.8). Results clearly show that the alkaloid content is significantly influenced by the soil pH but genotypic differences regarding the reaction to different pH values in the soil were observed.

1. Introduction

Lupins as protein crops can be used in many ways. They are grown for green manure, for animal feed, or for human nutrition. Unlimited feeding of bitter seeds led to the disease "lupinose" in former times. It was caused by the alkaloids contained in bitter lupins [1]. Only breeding of the so-called sweet lupins [2] facilitates the use of lupins to a greater extent in animal feed and for human nutrition. But besides genetics the alkaloid content in sweet lupins, for example, *Lupinus angustifolius,* is influenced by different environmental factors. Apart from drought, heat, and nutrient deficiencies, plants are largely affected by the soil pH [3]. Lupin species are differing concerning their demands for optimal growth, but in general commercial cultivars of lupins grow poorly on alkaline or neutral soils [4]. In general blue lupins (*Lupinus angustifolius*) and especially yellow lupins (*Lupinus luteus*) are less sensitive to calcareous soils than white lupins (*Lupinus albus*) [5, 6]. The soil pH considerably influences yield and protein content of *Lupinus angustifolius* [7, 8], that is, the higher the soil pH the lower the kernel yield and protein content. Besides this, the pH value has an impact on the production of secondary metabolites [9–11], for example, out of many soil parameters analysed, the highest correlation of the production of the glycoside salidroside in *Rhodiola sachalinensis* was observed to the soil pH [9]. Similar results were obtained for the alkaloid production, for example, when the pH value in cell culture media of *Lupinus polyphyllus* decreased from pH = 5.5 to pH = 3.5, the alkaloid production increased [12].

The objective of our field studies was to assess the influence of the soil pH on the alkaloid content of narrow-leafed lupin cultivars (*Lupinus angustifolius*).

2. Materials and Methods

2.1. Field Trials. Seeds of *Lupinus angustifolius* cultivars Borlana, Borweta, Bordako, Boruta, Borlu, Bora, Boregine, Boltensia, Bolivio, Vitabor, and Haags Blaue were supplied by the seed company Saatzucht Steinach (Bornhof, Germany). The variety Sonet was provided by Kruse Saatzucht (Münster, Germany).

In 2004 and in 2005, field experiments with 11 cultivars of *Lupinus angustifolius* (except Haags Blaue) were carried out under organic farming conditions in Bogen (Bavaria, northern latitude: 48.912925, eastern longitude: 12.692792) and in Gross Luesewitz (Mecklenburg-Western Pomerania,

Table 1: Characteristic of locations.

	Mecklenburg-Western Pomerania		Bavaria	
	Gross Luesewitz	Bornhof	Bogen	Gründl
Soil type	Loamy sand	Sand	Sandy clay loam	Sandy loam
pH value	5.8	5.3	7.2	6.7
Mean annual rainfall (mm)	620	558	803	822
Mean annual temperature (°C)	8.2	8.2	7.7	8.6

Source: [8, 13].

Table 2: Content of alkaloids in different cultivars of *Lupinus angustifolius* at different locations (2004 and 2005).

Genotype	Year	13-Hydroxylupanine		Angustifoline		Isolupanine		Lupanine	
		Bo	GL	Bo	GL	Bo	GL	Bo	GL
	2004	45.5	215.5	20.8	102.8	7.0	23.7	16.8	179.2
Bolivio	2005	52.2	157.8	41.8	87.3	10.2	26.6	6.6	204.7
	Mean	**48.9**	**186.7**	**31.3**	**95.1**	**8.6**	**25.2**	**11.7**	**192.0**
	2004	2.4	73	2.3	52.1	2.3	11.3	7.5	136.5
Boltensia	2005	1.8	58.1	1.5	50.5	0.0	16.8	1.2	216.8
	Mean	**2.1**	**65.6**	**1.9**	**51.3**	**1.2**	**14.1**	**4.4**	**176.7**
	2004	15.5	119.9	13.3	78.3	6.8	24.2	27.7	199.1
Bora	2005	2.1	94.3	0.6	72.1	1.0	34.4	4.5	293.8
	Mean	**8.8**	**107.1**	**7.0**	**75.2**	**3.9**	**29.3**	**16.1**	**246.5**
	2004	13.8	135.2	9.3	98.7	3.6	17.5	10.5	231.9
Bordako	2005	4.9	80.1	4.6	80.7	1.4	26.8	2.6	340.8
	Mean	**9.4**	**107.7**	**7.0**	**89.7**	**2.5**	**22.2**	**6.6**	**286.4**
	2004	12.7	67.6	7.4	46.3	4.6	18.8	10.5	113.6
Boregine	2005	1.8	75.5	0.8	66.7	0.5	33.6	0.3	245.7
	Mean	**7.3**	**71.6**	**4.1**	**56.5**	**2.6**	**26.2**	**5.4**	**179.7**
	2004	5.0	67.4	2.8	49.6	1.9	11.1	4.1	103.9
Borlana	2005	5.1	43.7	5.5	41.1	1.1	14.1	0.9	171.9
	Mean	**5.1**	**55.6**	**4.2**	**45.4**	**1.5**	**12.6**	**2.5**	**137.9**
	2004	12.6	118.6	5.7	79.5	3.3	18.8	9.6	151.2
Borlu	2005	3.8	36.4	5.3	31.6	1.5	11.2	5.5	106.3
	Mean	**8.2**	**77.5**	**5.5**	**55.6**	**2.4**	**15.0**	**7.6**	**128.8**
	2004	18.7	53.3	8.3	31.0	3.3	6.9	14.8	82.0
Boruta	2005	2.7	34.7	0.5	32.0	0.0	7.2	2.4	115.0
	Mean	**10.7**	**44.0**	**4.4**	**31.5**	**1.7**	**7.1**	**8.6**	**98.5**
	2004	11.2	119.2	6.4	87.0	3.0	12.4	17.3	167.4
Borweta	2005	5.0	19.5	2.9	23.9	0.0	8.2	4.2	136.7
	Mean	**8.1**	**69.4**	**4.7**	**55.5**	**1.5**	**10.3**	**10.8**	**152.1**
	2004	32.7	135.6	22.7	96.1	4.9	14.8	32.2	215.3
Sonet	2005	35.7	89.8	36.8	98.1	3.7	15.1	55.4	364.0
	Mean	**34.2**	**112.7**	**29.8**	**97.1**	**4.3**	**15.0**	**43.8**	**289.7**
	2004	4.5	19.9	2.0	11.3	1.7	3.4	3.0	25.7
Vitabor	2005	2.2	14.7	0.7	10.1	0.0	3.1	0.6	36.9
	Mean	**3.4**	**17.3**	**1.4**	**10.7**	**0.9**	**3.3**	**1.8**	**31.3**

BO: Bogen, GL: Gross Luesewitz.

Table 3: Mean content (±SD) of alkaloids in different cultivars of *Lupinus angustifolius* at different locations (2004 and 2005).

Alkaloid	Bogen		Gross Luesewitz	
13-Hydroxylupanine	13.3 ± 14.9	a	83.2 ± 50.5	b
Angustifoline	9.2 ± 11.5	a	60.3 ± 29.5	b
Isolupanine	2.8 ± 2.6	a	16.4 ± 8.9	b
Lupanine	10.8 ± 13.1	a	174.5 ± 87.6	b

Means for the same alkaloid between the locations with different letters are significantly different from each other (alpha = 0.05).

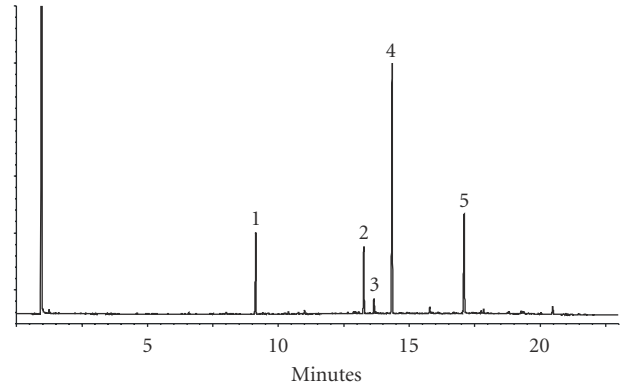

Figure 1: Gas chromatogram for different alkaloids (1 ISTD-Caffeine, 2 Angustifoline, 3 Iso-Lupanine, 4 Lupanine, 5 13-Hydroxy-Lupanine) present in cultivar Haags Blaue grown at Bornhof.

northern latitude: 54.071955, eastern longitude: 12.321031). Field experiments were conducted in a randomized block design with four replications with a plot size of 9.6 m^2. The alkaloid content in these trials was analysed on a mixed sample of each cultivar. In 2010 the variety Boruta and the variety Haags Blaue as well as six newly developed breeding lines (51–56) of the Saatzucht Steinach were grown in field experiments in fourfold replications in 4.2 m^2 plots at different soils at Gründl (Bavaria, northern latitude: 48.519305, eastern longitude: 11.816175) and Bornhof (Mecklenburg-Western Pomerania, northern latitude: 53.477371, eastern longitude: 12.911754) under conventional growing conditions. In this experiment the alkaloid content was measured separately for each plot. In Table 1 the characteristics of the locations are given.

2.2. Determination of the Alkaloid Content. Grain samples of about 250 g were randomly taken and grounded as described by [14]. All wholemeal samples revealed a dry matter content of about 90% and were stored at 20°C until analysis. The subsequent alkaloid analysis in lupin whole meal flour was carried out according to [14–16]. Main alkaloids were calculated as the sum of alkaloids shown in Figure 1. The main alkaloids in narrow-leafed lupins are angustifoline, isolupanine, lupanine, and 13-hydroxylupanine.

The determination of the alkaloid content was performed twice per sample with a coefficient of variation lower than 4%.

2.3. Statistical Analysis. To assess the effects of the location on the alkaloid content, a generalized linear model for the analysis of variance (ANOVA) was applied, using the GLM procedure of the software package SAS (version 9.3) followed by a Tukey test ($\alpha = 0.05$) for comparing the means. The two datasets were analysed separately.

3. Results and Discussion

The alkaloid content in sweet narrow-leaf lupin cultivars is in general very low. Already Sengbusch [17] suggested to call lupins alkaloid-poor (0.05% alkaloids in seeds) or alkaloid-free (0.025% alkaloids in seeds). Nevertheless, the seed alkaloid content of sweet lupins is influenced by different environmental factors such as fertilizers [18–22], ambient temperature during initiation of flowering up to pod ripening [14], and drought stress [23]. In 2004 and 2005, the mean daily temperature at the beginning of flowering until harvest in August was very similar [14]. The experiments were also carried out under uniform agronomic management (fertilizer, herbicides, etc.), so that the abiotic stress factors temperature, drought, and nutrient deficiency as well as mechanical damage can be neglected. In 2004 at Bogen the main alkaloid content of the cultivars tested ranged between 11.2 μg/g for the cultivar Vitabor and 92.5 μg/g for the cultivar Sonet and 90.1 μg/g for Bolivio. At Gross Luesewitz the alkaloid content ranged between 60.3 μg/g for cultivar Vitabor and 521.2 μg/g for cultivar Bolivio. The alkaloid content of all varieties tested is shown in Table 2. In 2005, as expected, the lowest alkaloid content was also found in the cultivar Vitabor (Bogen 3.5 μg/g and Gross Luesewitz 64.8 μg/g) and the highest content in Sonet (Bogen 131.6 μg/g and Gross Luesewitz 567.0 μg/g).

Out of all alkaloids analyzed the lupanine content shows the largest increase with decreasing soil pH. Christiansen et al. [23] reported that drought stress during the vegetative phase reduces mostly the concentration of lupanine, 13-hydroxylupanine, and angustifoline, whereas isolupanine is affected to a much smaller extent.

The differences in the alkaloid content between the locations in Mecklenburg-Western Pomerania and Bavaria are significant (Table 3).

Figure 2 clearly demonstrates that the alkaloid production is significantly higher when lupins are cultivated at a lower pH (mean value of two years is shown). However, also clear differences are observed between cultivars opening the opportunity to breed cultivars with low alkaloid content under low pH conditions, for example, Vitabor.

In 2010 also two cultivars and six newly developed breeding lines of *Lupinus angustifolius* were analyzed concerning the alkaloid content at locations with different soil pH. At Gründl (pH = 6.7) the total alkaloid content ranged between 0.0166% and 0.1293% while at Bornhof (pH = 5.3) the alkaloid content was in general higher and ranged between 0.029% and 0.1810% (Figure 3). The variety Boruta and

Table 4: Mean alkaloid content estimated on soil with pH = 5.8 and pH = 7.2 observed in cultivars of *Lupinus angustifolius* at different locations (2004 and 2005, $n = 22$).

Location	Main alkaloid content [%]	
	2004	2005
Mecklenburg-Western Pomerania (pH = 5.8)	0.0330	0.0339
Bavaria (pH = 7.2)	0.0043	0.0029
Least significant difference (Tukey, $\alpha = 0.05$)	0.0084	0.01

Table 5: Mean alkaloid content estimated on soil with pH = 5.3 and pH = 6.7 observed in actual varieties and breeding lines of *Lupinus angustifolius L.* at different locations (2010, $n = 32$).

Location	Alkaloid content [%]
	2010
Mecklenburg-Western Pomerania (pH = 5.3)	0.0687
Bavaria (pH = 6.7)	0.0355
Least significant difference (Tukey, $\alpha = 0.05$)	0.0081

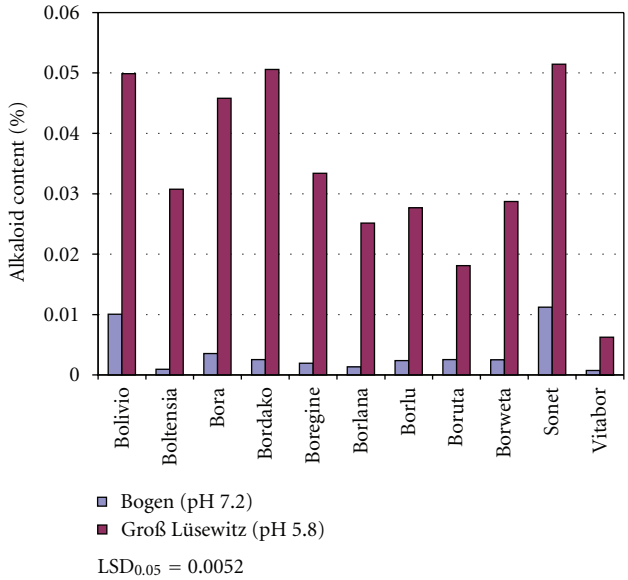

Figure 2: Influence of the soil pH on the main alkaloid content of *Lupinus angustifolius* (mean of 2004 and 2005).

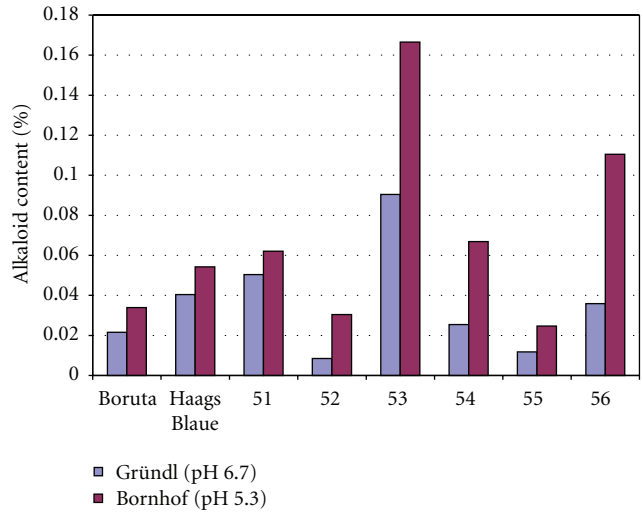

Figure 3: Influence of the soil pH on the main alkaloid content of *Lupinus angustifolius* cultivars and breeding lines (2010).

two new breeding lines revealed the lowest alkaloid content (Figure 3).

Concerning the average main alkaloid content in cultivars and breeding lines significant differences between locations were observed in 2010 (Table 5) as it was previously observed in 2004 and 2005 (Table 4). In Germany, there is no law concerning the upper threshold of alkaloids in lupins for animal feed and human nutrition, but in general the upper threshold for use in animal and human nutrition is 0.05% and 0.02%, respectively. In 2010 this threshold has been exceeded in general at the soil with the lower pH (Mecklenburg-Western Pomerania) and also by some cultivars in 2004/2005.

For lupin cultivation a rather low soil pH (pH = 5.0–6.8) is recommended [5]. Jansen et al. [16] reported that the grain yield at pH = 7.2 is lower than at pH = 5.8. On the other hand the alkaloid content of *Lupinus angustifolius* decreases significantly at a higher pH (Figures 2 and 3). Therefore, for a high yield combined with low alkaloid content, the soil pH is of prime importance, although it has to be taken into account that also additional environmental factors, for example, drought stress, fertilization, and temperature, can have an adverse effect on the alkaloid content in lupin seeds. Yaber Grass and Leicach [24] noted that a significant increase in the total alkaloid content was observed from samples of *Senecio grisebachii* growing in highly deteriorated soil compared to those from samples grown in less deteriorated ones.

The results presented show that additional breeding efforts are needed to achieve low alkaloid content also under low soil pH. As shown in Figures 2 and 3 genetic variation concerning this trait is present in cultivars and new breeding lines which could be exploited in the future.

Acknowledgments

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Research Article

Growth and Physiological Responses of *Phaseolus* Species to Salinity Stress

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This paper reports the changes on growth, photosynthesis, water relations, soluble carbohydrate, and ion accumulation, for two salt-tolerant and two salt-sensitive *Phaseolus* species grown under increasing salinity (0, 60 and 90 mM NaCl). After 20 days exposure to salt, biomass was reduced in all species to a similar extent (about 56%), with the effect of salinity on relative growth rate (RGR) confined largely to the first week. RGR of salt-tolerant species was reduced by salinity due to leaf area ratio (LAR) reduction rather than a decline in photosynthetic capacity, whereas unit leaf rate and LAR were the key factors in determining RGR on salt-sensitive species. Photosynthetic rate and stomatal conductance decreased gradually with salinity, showing significant reductions only in salt-sensitive species at the highest salt level. There was little difference between species in the effect of salinity on water relations, as indicated by their positive turgor. Osmotic adjustment occurred in all species and depended on higher K^+, Na^+, and Cl^- accumulation. Despite some changes in soluble carbohydrate accumulation induced by salt stress, no consistent contributions in osmotic adjustment could be found in this study. Therefore, we suggest that tolerance to salt stress is largely unrelated to carbohydrate accumulation in *Phaseolus* species.

1. Introduction

Salinity is considered a significant factor affecting crop production and agricultural sustainability in arid and semiarid regions of the world, reducing the value and productivity of the affected lands [1]. Because soil infertility is often due to the presence of large amount of salt, the identification of plants capable of surviving under these conditions is worth investigating [2]. Currently, there are no economically viable technological means to facilitate crop production under salt stress conditions. Nevertheless, development of genotypes with field tolerance to salinity stress is considered a promising approach, which may help to satisfy growing food demands of developed and developing countries. To improve on salt stress tolerance requires knowledge of the physiological mechanisms and genetic controls of the traits associated with salt tolerance at different plant development stages.

To understand the physiological mechanisms responsible for salinity tolerance, it is necessary to know whether their growth is limited by the osmotic effect of the salt in the soil, or by the toxic effect of the salt within the plants. In the simplest analysis of the response of a plant to salinity stress, the reduction in shoot growth occurs in two phases: a rapid response to the increase in external osmotic pressure, and a slower response due to the accumulation of Na^+ in leaves [1]. In the first *osmotic phase* which starts immediately after the salt concentration around the roots increases to a threshold level (40 mM NaCl for most plants, which is equivalent to ECe of 4 dS/m; [3]), the rate of shoot growth falls significantly. This is largely due to the osmotic effect of the salt outside the roots. The second, *ion-specific*, phase of plant response to salinity starts when salt accumulates to toxic concentrations in the leaves, causing necrosis and reducing the photosynthetic area, resulting in further decline of growth [1, 4].

In the past 2 decades, biotechnology research has provided considerable insights into the mechanism of abiotic stress tolerance in plants at the physiological and molecular levels [4]. Stress tolerance mechanisms may vary from species to species and at different developmental stages [5]. Salt tolerance in crops is based on specific physiological characteristics like shoot or leaf specific ion accumulation or production of specific osmolyte compounds [2].

Ion transport processes are central to the understanding of the complex and multigenic nature of salt tolerance in crop plants [2]. The crucial role of K^+ homeostasis in salt tolerance mechanisms of salinized plants have placed it in center stage [6, 7]. Imposition of salt stress results in a massive efflux of K^+ from cells [8] and significantly reduces the intracellular pools of K^+ [9]. Mitigation of this loss strongly correlates with the level of salt tolerance [8, 10–12].

Plant abiotic stress-tolerance is often associated with increased *de novo* synthesis of so-called compatible solutes [4]. Traditionally, the role of osmolytes in drought and salt tolerance was thought to be as cytosolic osmoticum involved in cellular osmoregulation [4]. However, the measured levels of many compatible solutes often appear to be too low to act as osmolytes [13]. It has been proposed that the role of compatible solutes in cytosolic osmotic adjustment is indirect, through regulatory or osmoprotective functions. The latter may include a possible role for compatible solutes in stabilizing the structure and activities of enzymes and protein complexes, scavenging radical oxygen species and maintaining the integrity of membranes under dehydration stress conditions [2, 14]. Another function of compatible solutes may be in maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cells [10].

In plants, growth is particularly important because survival and reproduction depend on plant size and therefore on growth rate. Relative growth rate (RGR) is therefore a key variable when comparing plant species growing under stressful environments [15]. RGR is determined by two factors, the unit leaf rate (ULR), which is an index of plant photosynthetic-assimilatory capacity per leaf area unit, and leaf area ratio (LAR), which is the amount of leaf area per total plant weight [16]. In some species, salinity mainly affects the leaf elongation and hence the development of photosynthetic surface area (LAR) [17] and photosynthetic capacity in others [18]. Salinity reduction of LAR could be caused by a decrease in SLA (the amount of leaf area per unit leaf weight) and/or a decrease in the proportion of dry matter allocated to the leaf tissue (leaf weight ratio) (LWR) [16]. At the whole plant level, these growth parameters may make it possible to clarify whether genotypic variation in salt tolerance could be attributed to morphological or photosynthetic response [15, 19].

The common bean, *Phaseolus vulgaris* L., is extremely sensitive to salinity, and suffers yield losses at soil salinity of less than $2\,dS\,m^{-1}$ [20]. However, the common bean is regarded as an appropriate crop for bioproductivity enhancement and marginal land reclamation, not only because it yields nutritious fodder, protein rich seeds, but also it is a soil nitrogen enricher in symbiotic association with rhizobium [21]. Common bean is known to exclude Na^+ from the leaves, but takes up Cl^- in proportion to the external concentration [20]. High leaf Cl^- concentrations reduce growth by altering the nutritional balance of the plant, affecting CO_2 assimilation [22, 23], and altering water relations [24]. Although there are several studies demonstrating the effect of salinity on bean growth [25, 26], there is limited genetic variation in cultivated bean germplasm for salinity tolerance [24].

Certain *Phaseolus* species such as wild *P. acutifolius* Gray var. *latifolius* Freem. and *P. vulgaris* L. can be classified as salt tolerant due to their ability to restrict Na^+ ions in roots and leaves [23]. Salt tolerance in both species is also associated with better stomatal control through osmotic adjustment. *Phaseolus* species adjust to high salt concentrations by lowering tissue osmotic potential with an increase of inorganic ions, predominantly Cl^-, Na^+, and K^+ in their leaves [23]. The role of compatible solutes (e.g., soluble sugars) as possible osmolytes have not been well established or discarded on *Phaseolus* species. Many studies have dealt with osmotic potential decrease in common bean as a result of water deficit in the leaf tissue [26], but few differences between the various inorganic ions and compatible organic solutes contributing to osmolyte accumulation [24]. This allows the following hypothesis; assuming that the production of organic solute requires considerable expenditure of energy while accumulation of inorganic ions is inexpensive, it is possible that the ability of *Phaseolus* species to withstand osmotic stress can be attributed to changes in the ratio of organic and inorganic compounds that contribute to osmotic adjustment. Therefore, the object of this study was to evaluate the effects of salt stress on growth, water relations, and gas exchange of different *Phaseolus* species, and at the same time correlate these effects with changes in ionic and soluble carbohydrate accumulation, to better understand the mechanisms of salt tolerance in these species.

2. Materials and Method

2.1. Plant Material and Location. Two wild *Phaseolus* genotypes, *P. vulgaris*, PI325687, *P. acutifolius*, G40169 and two cultivated genotypes, *P. vulgaris*, G04017 and *P. acutifolius*, G40142 were used. These genotypes were classified into three groups: salt-tolerant *P. vulgaris*, PI325687 (PvWT), moderately tolerant *P. acutifolius*, G40142 (PaCT) and salt-sensitive *P. acutifolius*, G40169 (PaWS) and *P. vulgaris*, G04017 (PvCS) based on the ranking in terms of variation on their salinity tolerance defined by total dry weight reduction as a percentage of the unsalinized controls, salt susceptibility index (SSI), and root: shoot ratio (RSR) [27]. Wild species were selected as they are widely distributed throughout the Pacific slopes of Mexico, where saline soils are common. The cultivated *P. vulgaris* is a Brazilian variety "Carioca" (G04017) belonging to the Mesoamerican gene pool with an indeterminate prostrate growth habit (Type III) and small seeds ($\leq 300\,mg\,seed^{-1}$) [21]. The cultivated *P. acutifolius* is grown in semiarid areas of Sonora, Mexico and also has small seeds ($\leq 137\,mg\,seed^{-1}$). Plants were grown on nutrient solution under greenhouse conditions.

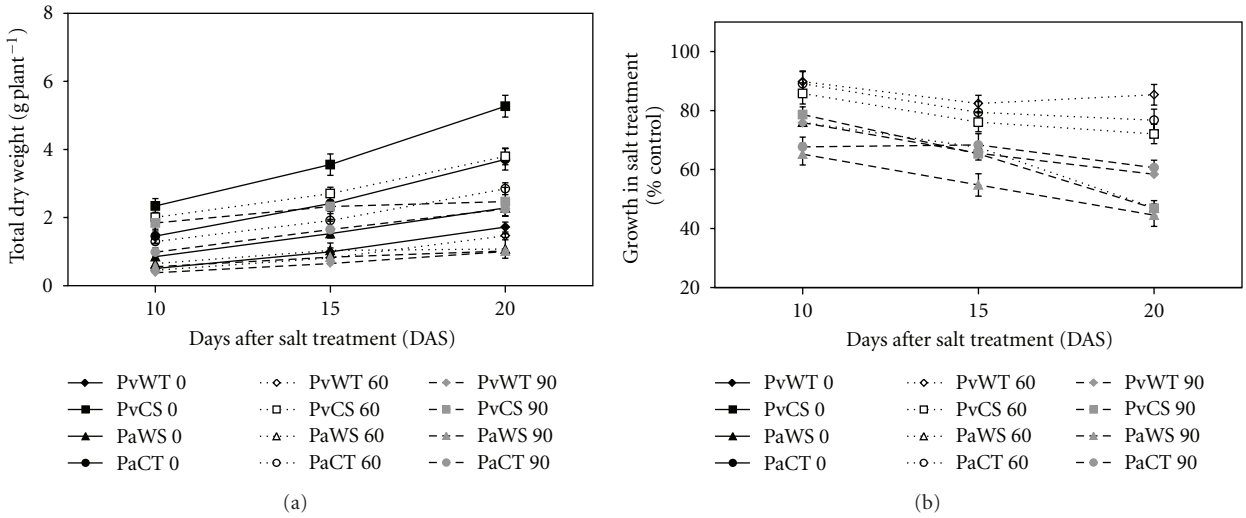

Figure 1: Effects of 0, 60, and 90 mM NaCl on total dry weight (a) and percentage total dry weight (b) in salt relative to control conditions between 10 and 20 days after salt treatment for *P. vulgaris* PI325687 (PvWT), *P. vulgaris* G04017 (PvCS), *P. acutifolius* G40169 (PaWS), and *P. acutifolius* G40142 (PaCT). Each value represents the mean ± SE of six replicates.

Average temperature during the experiment was 26°C, and minimum and maximum temperatures were 22°C, and 34°C, respectively. Relative humidity varied between 50 and 65%.

2.2. Plant Growth. Seeds were surface sterilized with 2.5 g L^{-1} sodium hypochlorite for 5 min and rinsed with sterile distilled water, then they were scarified mechanically and germinated in the dark at 25°C in rolled germination paper (Anchor Paper Co., St. Paul, MN) moistened with 0.5 mM $CaSO_4$. Seven-day-old seedlings of uniform size were transferred to aerated tanks (100 L) containing nutrient solution [28]. Nutrient solution composition, in mM, was: 6 KNO_3, 4 Ca $(NO_3)_2$, 1 $MgSO_4$, 1 $NH_4H_2PO_4$, 0.05 Fe-EDTA, 0.05 KCl, 0.025 H_3BO_3, 0.002 $MnSO_4$, 0.002 $ZnSO_4$, 0.005 $CuSO_4$, 0.005 $(NH_4)_6MoO_2$ $4H_2O$. The solution pH was adjusted daily to 6–6.5. The nutrient solution was aerated continuously and replaced weekly. Plants were grown in this control solution until the emergence of the first trifoliate leaf (7 days after transplanting), at which time salt treatments were added to the solutions. The treatment nutrient solution was identical to that for controls except for the addition of NaCl. Plants were exposed gradually to their final NaCl concentration (0, 60 and 90 mM) through a progression of 30 mM NaCl increments at one-day intervals added shortly before sunset. A randomized complete block design with a split-plot arrangement of treatments and six replications was used with NaCl treatments as the main plot and genotypes as subplots.

2.3. CO_2 Assimilation and Stomatal Conductance. Measurements of net CO_2 assimilation (A_n) and leaf diffusive conductance (g_s) were taken at 9, 14, and 19 days after initiation of salt treatments (DAS) using the second, third, and fifth trifoliate leaf, which were the youngest fully expanded leaves, respectively. Measurements were performed using a LI-COR 6400 infrared gas analysis system (LI-COR, Corp., Lincoln, NE). A portion of the central leaflet was enclosed in a ventilated temperature controlled leaf chamber (6 cm^2). A_n was measured at 34 MPa external CO_2 partial pressure (340 μ mol CO_2 mol^{-1} air) and a vapor pressure deficit (VPD) of 1.8 KPa. The photosynthetic photon flux density (PPFD) was 1200 μ mol m^{-2} s^{-1}, provided by a 6400-02 LED light source. Gas exchange rates were monitored until steady-state rates were attained.

2.4. Leaf Water Relations. Predawn water potential (Ψ_w) of a whole leaf was measured with a pressure chamber (Model 3000, Soilmoisture, Santa Barbara, CA) [29]. Leaf osmotic potential (Ψ_π) measurements were made on the remainder leaf material that was used for leaf water measurements. Osmotic potential (Ψ_π) was determined by pressing frozen thawed tissue with a ground plastic tissue homogenizer. The homogenate was centrifuged for 5 min at 2000 ×g in an Eppendorf micro centrifuge and 10 μL of supernatant was collected for measuring leaf solute potential with a Wescor-5500 vapor pressure osmometer (Wescor, Logan, UT, USA). The osmometer was recalibrated after every pair of readings using commercial standards. Readings were converted to pressure units by using the van't Hoff equation ($\pi = -cRT$), where c is osmolality (mOsmol kg^{-1}), R is the gas constant, and T is temperature (°K). Turgor potential (Ψ_p) was determined as the difference between leaf water potential and osmotic potential. As a measure of osmotic adjustment to salt, a value for osmotic potential at full turgor Ψ_π was calculated as the product of the measured values and relative water content. This accounts for the effects of change in tissue hydration on leaf Ψ_π.

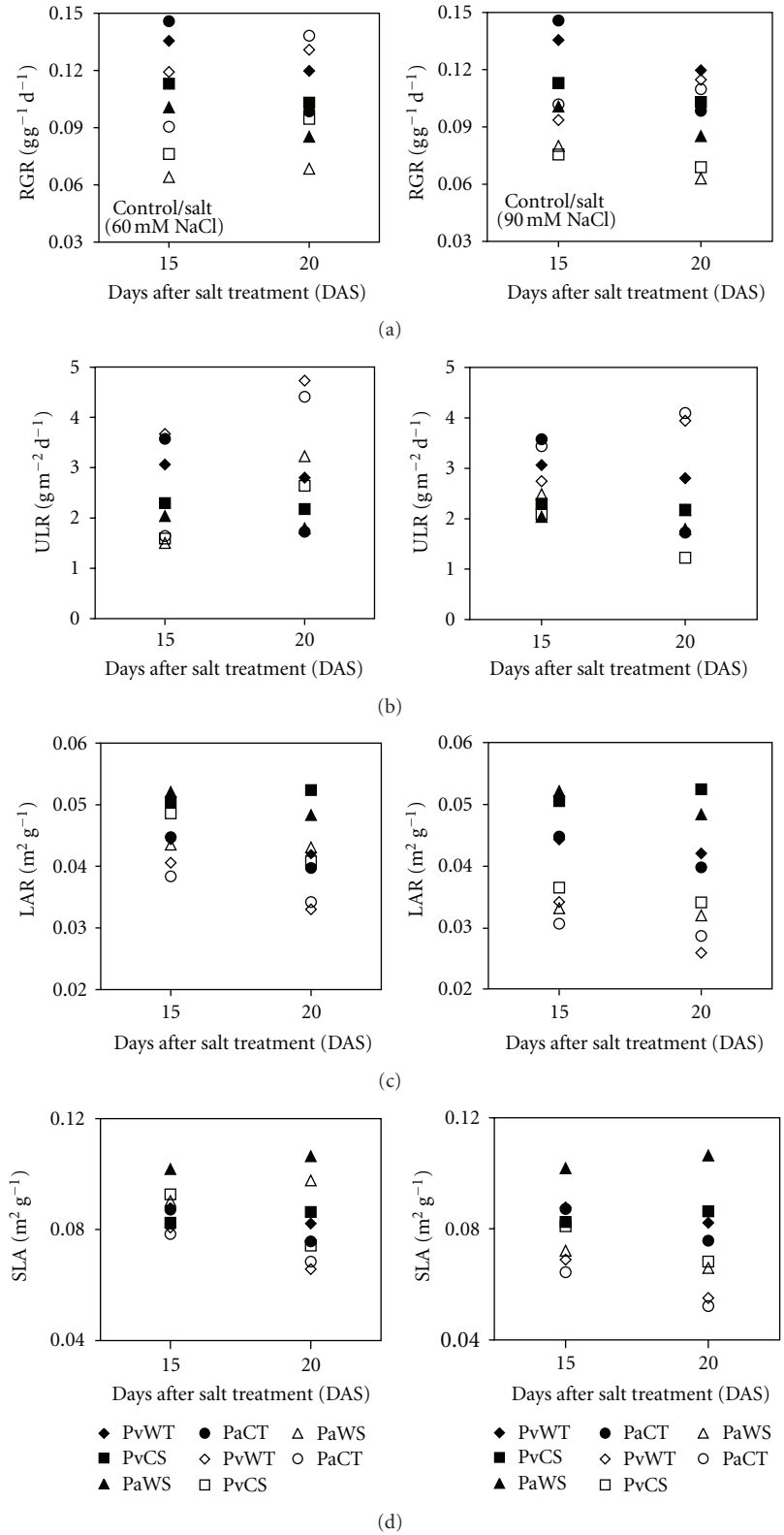

Figure 2: Effects of increasing NaCl levels on relative growth rate (RGR) (a), unit leaf rate (ULR) (b), leaf area ratio (LAR) (c), and specific leaf area (SLA) (d) for *Phaseolus* species, based on the differences between two means (10–15 and 15–20 days). Each value represents the mean ± SE of six replicates. Control (filled symbols) and salt treatments open (symbols).

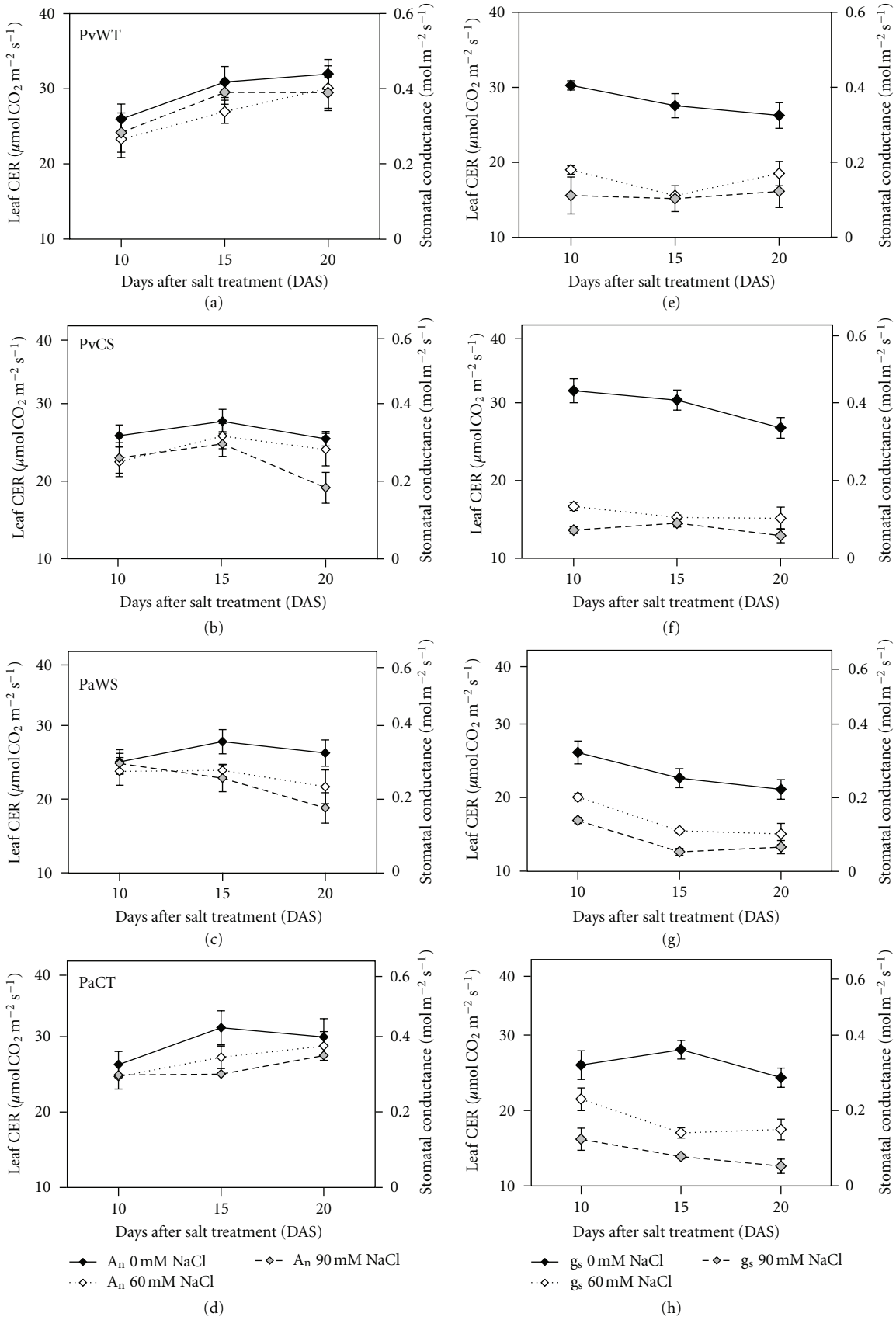

FIGURE 3: Effects of increasing NaCl levels on leaf CO_2 exchange rate (*left axis*; a–d) and stomatal conductance (*right axis*; e–h) between 10 and 20 days of salt treatment for *Phaseolus* species. Each value represents the mean ± SE of six replicates.

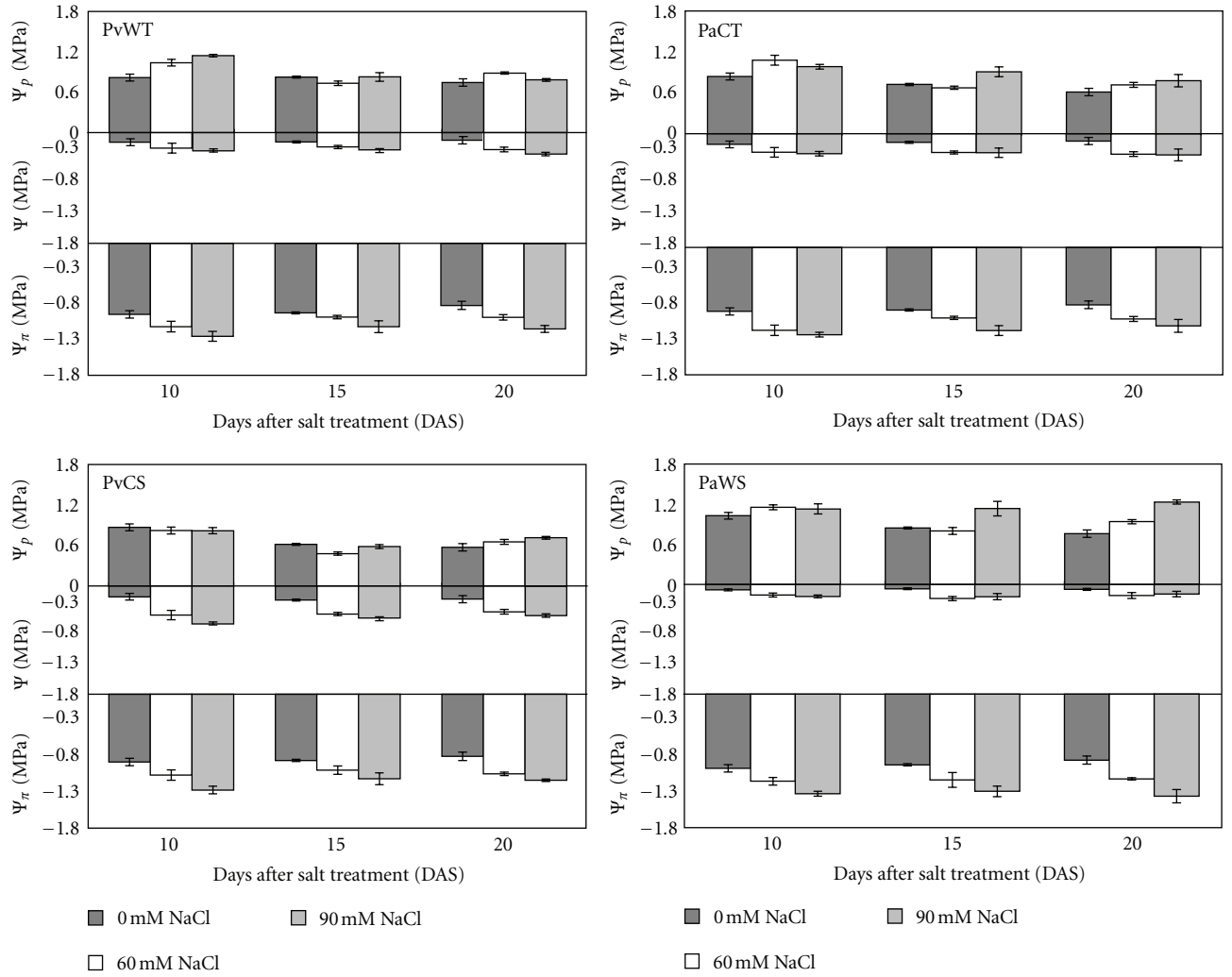

Figure 4: Effects of increasing NaCl levels on leaf turgor pressure (Ψ_p), leaf water potential (Ψ), and osmotic potential (Ψ_π) (in MPa) between 10 and 20 days of salt treatment for *Phaseolus* species. Each value represents the mean ± SE of six replicates.

2.5. Growth Measurements. Plants were harvested at 10, 15, and 20 days after the initiation of salt treatments (DAS) and separated into roots, stem, and leaves. Plant material was dried at 65°C for 96 hours to determine dry weight. Leaf area was measured with a portable leaf area meter (Model LI-3000 A, LI-COR, Lincoln, NE). Growth parameters were calculated according to Hunt [16]. The mean relative growth rate, RGR ($g\,g^{-1}\,d^{-1}$), was calculated as was the rate of increase of total dry weight per unit for each period. Two growth components were determined: the unit leaf rate, ULR (also called net assimilation rate, $g\,m^{-2}\,d^{-1}$), calculated as the rate of increase of total dry weight per unit of total leaf area, and the leaf area ratio, LAR ($m^2\,g^{-1}$), calculated as the ratio between the total leaf area and the total plant dry weight. The leaf weight ratio, LWR ($g\,g^{-1}$), was calculated as the ratio between the total leaf dry weight and the total plant dry weight; and the specific leaf area, SLA ($m^2\,g^{-1}$), was calculated as the mean area of leaf displayed per unit of leaf weight, the RGR being related to these quantities by the equation: RGR = ULR × LAR = ULR × LWR × SLA.

2.6. Elemental Analysis. Tissue was ashed at 500°C for 8 h, followed by dissolution in 1 mM hydrochloric acid [30]. Sodium and potassium concentrations were determined by flame emission using an Atomic Absorption Spectrometer (Varian SpectrAA-220FS; Mulgrave, Australia). Free chloride was extracted from 3 mg of ground material with 50 mL of deionized water and then filtered through 0.22 μm millipore paper [31]. Chloride concentration was determined colorimetrically using an UV/BIS Spectrometer (Lamda 40 Perkin Elmer; Uberlingen, Germany).

2.7. Carbohydrate Analysis. Root, stem, and leaves samples were frozen with liquid nitrogen before storage at −20°C. An enzymatic assay method for nonstructural carbohydrate was used [32]. Soluble sugars were extracted from 15 mg of fine ground plant powder, in 4 mL methanol: water solution, followed by 100 μL chloroform. Two liquid phases were separated from the plant powder after centrifugation (IEC Model GP8R., Needham, MA). After evaporation under

vacuum (CentriVap Labcondo Model 75100, MO, USA), the dried pellet was returned to its soluble form by agitation in water at 4°C. The aqueous extract was then combined with 15 mg polyvinylpyrrolidone (PVP) to eliminate any residual phenols. After repeated shaking, the supernatant was analyzed using the MP plate (The Multtiskan Ascent MP Systems, Labsystems Thermo Fisher Scientific, Helsinki, Finland). Glucose, fructose, and sucrose concentrations were quantified by measuring the production of NADH.

2.8. Statistical Analysis. Prior to analysis of variance, salt treatments data for each variable were analyzed for normality and homocedasticity (homogeneity of covariance matrices) by using Bartlett's tests [33]. Because error variances of some variables were not homogenous, the data was transformed to natural logarithm, root square, or the inverse value. Original or transformed data were further subjected to parametric procedures when both requirements were met. Data was analyzed using the GLM procedure of the Statistical Analysis System [33]. Six replicates per salinity treatment per species per harvesting date and organ tissue were used for analyses of variables. Two-way analysis of variance was used to determine significant differences among species for various traits.

3. Results

3.1. Effect of Salt Stress on Growth. Growth of all four genotypes was reduced by a similar extent by salinity (Figure 1). Biomass production in the absence of salinity differed among genotypes, but the effect of salinity was similar, so that the genotypes that grew most in the control treatment also grew best in the salt treatment (Figure 1(a)).

Salt tolerance is shown in Figure 1(b), as the percent biomass in saline *versus* control conditions. This illustrates that the greatest reduction in plant growth occurred during the first period of salt treatment. There were no significant differences between genotypes, except during the first two harvests when PaWS were significantly more affected than PvCS as a percent of biomass. The final biomass production, after 20 d of salinity, was reduced by 47 to 72% for salt-sensitive genotypes and by 58 to 61% for salt-tolerant ones.

Calculations of total RGR throughout the experimental period showed that plants treated with 60 mM NaCl reduced grow only during the first period (10–15 DAS; Figure 2(a)). For subsequent harvest, there were statistically significant differences ($P \leq 0.0084$) in RGR between genotypes. RGR of salt-tolerant PvWT and PaCT was maintained between 15 and 20 days, whereas in salt-sensitive PvCS and PaWS, RGR declined with increasing salinity (Figure 2(a)). Thus, the effect of salt on final biomass was attributed to less growth in the first weeks of salt treatment.

ULR also declined over time in salt-sensitive genotypes, particularly in salinized plants (Figure 2(b)). For salt-tolerant genotypes (PvWT and PaCT), ULR was maintained across salt treatments and time (Figure 2(b)). LAR was steady during the first period (10–15 days) of salt stress, whereas LAR decreased for all genotypes with increasing salinity at day 20 (Figure 2(c)). Also LWR values were steady over time in all salt treatments and genotypes (data not shown), whereas SLA was significantly decreased ($P \leq 0.0007$) for all salt-stressed genotypes at 90 mM NaCl (Figure 2(d)).

3.2. Photosynthesis and Stomatal Conductance. Salinity and salt stress duration significantly affected photosynthesis (A_n) ($P \leq 0.0025$) and stomatal conductance (g_s) ($P \leq 0.0001$). Salinity and species interaction was not significant ($P \leq 0.1903$), indicating that all species responded similarly to salt stress. A_n was steady with time in control plants throughout the experiment period and decreased in the salt treatment (90 mM NaCl) only in salt-sensitive genotypes (Figures 3(a)–3(d)). No significant differences were detected on stomatal conductance for all genotypes under nonsaline conditions throughout the experiment but declined as salinity and duration intensified (Figures 3(e)–3(h)).

3.3. Ions. Tissue concentration of Cl^- and Na^+ ions increased significantly in response to salt treatments (Table 1). However, the magnitude of the Cl^- increments was always higher than those of Na^+ at all salt treatments. The concentrations of Na^+ increased in plants treated with salt stress until day 15 (0.79 to 1.06 mmol kg^{-1} DW), and then remained constant until day 20 (Table 1), whereas the concentration of Cl^- in plants treated with 60 and 90 mM NaCl increased sharply between day 10 and 20 (1.75 to 2.44 and 1.63 to 5.24 mmol kg^{-1} DW), except in PaCT.

Saline-induced changes in minerals concentration varied with plant organ and ion. In all species, Na^+ concentration increased almost equally in stems and roots, whereas the concentration of Cl^- increased more in stems and leaves than in roots (Table 1). Species differed in leaf Na^+ accumulation. PvWT and PaCT were able to exclude Na^+ from leaves at 60 mM NaCl. In contrast, PaWS accumulated Na^+ in their leaves as salt levels increased (Table 1). Salinity reduced K^+ concentration in the root, stems and leaves of all species (Table 1). However, decrease in K^+ concentration on stems of PvWT and PvCS was greater than leaves and roots (Table 1). Compared to controls, leaf and root K^+ concentration at 20 days with 90 mM NaCl decreased between 24 to 46 and 40 to 72%, respectively. At moderate and high salinity levels, leaf K^+ concentration on PaWS and PaCT was about 28 to 15% higher at day 20 than those observed on PvWT and PvCS genotypes.

3.4. Carbohydrates. Hexoses (glucose plus fructose) of both PvWT and PvCS decreased proportionally to the amount of salt added to the nutrient solution (Table 2). In both PaWS and PaCT, the increase in hexose concentration was linear over salt treatment, attaining values about three times as high as those in control plants, particularly at day 10 (data not shown). Sucrose concentration was also affected by salinity where sucrose of PvWT and PvCS decreased linearly with salt treatments (Table 2). In PaWS and PaCT, however, no significant differences were detected under both salinity levels.

Table 1: Effects of external NaCl concentrations on mineral composition of leaves, stem, and roots of *Phaseolus* species.

Species/Genotype	Leaves			Stem			Root		
NaCl (mM)	mmol kg^{-1} dry weight								
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
P. vulgaris PvWT									
0	17 c[z]	1382 a	9 c	27 c	1308 a	16 c	20 b	1302 a	29 c
60	13 b	1021 b	1078 b	222 b	1077 b	658 b	423 a	838 b	663 b
90	159 a	747 c	2153 a	285 a	831 c	1342 a	460 a	778 b	999 a
P. vulgaris PvCS									
0	20 b	1289 a	2 c	22 b	2267 a	2 c	21 c	1403 a	25 c
60	143 a	849 b	767 b	276 a	1527 b	672 b	321 b	806 b	481 b
90	154 a	727 b	1895 a	268 a	1272 c	1262 a	480 a	703 b	879 a
P. acutifolius PaWS									
0	19 c	1430 a	23 a	24 c	1428 a	32 c	18 b	1773 a	45 c
60	166 b	1213 b	933 b	257 b	877 b	443 b	377 a	901 b	452 b
90	223 a	1032 b	1889 c	309 a	704 c	880 a	447 a	495 c	1040 a
P. acutifolius PaCT									
0	14 c	1222 a	22 c	22 b	1188 a	26 c	15 b	2158 a	34 c
60	90 b	952 b	1109 b	289 a	1073 a	418 b	349 a	905 b	630 b
90	267 a	926 b	2201 a	256 a	612 b	843 a	371 a	788 b	965 a
F-values from ANOVA									
NaCl	470***	112***	79***	352***	89*	37***	308***	118***	1111***
Species	12***	17***	13***	0.1[ns]	109***	18***	7**	13.6***	15***
NaCl × Species	29***	3.3**	3.5**	5.1**	7.7***	6.9***	4.7**	11.2***	9.1**

[z]Values are means of six replicates after 20 days of salt exposure. Differences among treatments at $P \leq 0.05$ are given according to Duncan multiple range test. ns: not significant, *, **, *** Significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Saline-induced changes in soluble carbohydrate concentration were also highly dependent upon the species and plant organ (Table 2). Hexose accumulation on leaves and stems occurred during the first 10 days of salinization in all species (data not shown). However, these accumulations decreased over time in PvWT and PvCS, with the lowest content at day 20 (Table 2). In PaWS and PaCT, similar hexose concentrations were found in leaves and stems in both salt treatments, with the highest concentration at day 20 (Table 2). Hexose concentration in roots also increased with salinity in all species except for PvCS. Sucrose concentration sharply increased in relation to stress intensity and duration in the leaves and stems in PaWS and PaCT at day 15 (data not shown), and remaining constant for the remainder of the study (Table 2). On PvCS, however, sucrose accumulation decreased with time and salinity stress.

3.5. Plant Water Relations. Differences in leaf turgor, water, and osmotic potentials among genotypes were statistically significant at all salt concentration ($P \leq 0.0001$) (Figure 4). All genotypes except PvWS had significantly higher leaf water potentials at increasing NaCl concentrations for most of the experimental period ($P \leq 0.0001$) (Figure 4). Differences on leaf osmotic potential among genotypes were observed only for high salinity at day 20. Generally, the leaf osmotic potential decreased as salt level increased. Osmotic potential of all species ranged from −0.92 to −1.4 MPa for salt treatments. Leaf turgor potential increased between 0.47 and 0.95 MPa at 60 mM NaCl and between 0.70 and 1.2 MPa at 90 mM NaCl (Figure 4).

At the end of the experiment, all genotypes showed significant differences between control and salt-stressed plants in ions and soluble carbohydrates to Ψ_π (Table 3). Among the genotypes, PaWS had the lowest Ψ_π due to ions in both control and high salt stress (Table 3). This genotype had the highest leaf K^+ and Na^+ concentration in the control and salt treatments (Table 1), which contributed to its having the lowest Ψ_π (Table 3). In all genotypes, inorganic ions accounted for approximately 60% of total Ψ_π in control plants. With salt treatment (60 and 90 mM NaCl), the relative contribution from ions remained near 71 and 82% for the salt-tolerant genotypes, whereas it accounted to about 60 and 85% for the salt-sensitive genotypes.

In regard to the degree of osmotic adjustment due to the three ions (Na^+, K^+, and Cl^-), the salt-sensitive genotypes PaWS showed the highest increase in Ψ_π due to ions, 0.32 MPa (Table 3). The other salt-sensitive genotype, PvCS, was quite different, and had the least increase in Ψ_π due to ions, 0.16 MPa. In the salt-sensitive genotype PvCS, the osmotic adjustment due to ions made up about half the total osmotic adjustment (48%), whereas it accounted to about 76% in PaWS (Table 3). In contrast, in salt-tolerant genotypes, the change in Ψ_π due to ions was lower than the change in total Ψ_π (Table 3). Thus, ions made up 80% of the total osmotic adjustment. The salt-induced soluble

Table 2: Effects of external NaCl concentrations on glucose (Glu), fructose (Fru), and sucrose (Suc) of leaves, stem, and roots of *Phaseolus* species.

Species/Genotype	Leaves			Stem			Roots		
NaCl (mM)	mmol kg^{-1} dry weight								
	Glu	Fru	Suc	Glu	Fru	Suc	Glu	Fru	Suc
P. vulgaris PvWT									
0	4 b[z]	5 a	46 a	10 a	15 b	64 a	5 c	8 b	32 b
60	8 a	14 b	38 b	10 a	21 a	51 b	9 b	26 a	38 a
90	1 c	1 c	4 c	3 b	3 c	6 c	13 a	30 a	35 a
P. vulgaris PvCS									
0	6 b	7 b	42 a	19 b	25 b	50 a	5 b	8 b	50 a
60	17 a	22 a	33 b	36 a	44 a	39 b	16 a	34 a	27 b
90	2 c	2 b	3 c	11 b	15 c	6 c	2 b	4 b	4 c
P. acutifolius PaWS									
0	12 c	12 b	41 a	12 b	13 c	47 a	4 c	12 c	48 a
60	22 b	28 a	31 b	15 b	22 b	50 a	10 b	35 b	32 b
90	25 a	29 a	36 b	22 a	31 a	44 a	14 a	50 a	37 b
P. acutifolius PaCT									
0	10 b	12 b	34 a	22 a	24 a	47 a	8 b	15 c	51 a
60	24 a	32 a	30 b	21 a	25 a	38 b	8 b	33 b	30 c
90	24 a	31 a	29 b	21 a	26 a	41 b	15 a	52 a	38 b
F-values from ANOVA									
NaCl	56.9***	57.4***	576***	3.9[ns]	8.8*	261.9***	49.8***	91.2***	77.7***
Species	11.8***	8.5***	2.6*	15.3***	5.4***	9.1***	4.2**	13.3***	4.7**
NaCl × Species	2.3*	1.8[ns]	6.7***	6.7***	3.5**	16.4***	16.2***	9.5***	6.9***

[z]Values are means of six replicates after 20 days of salt exposure. Differences among treatments at $P \leq 0.05$ are given according to Duncan multiple range test. ns: not significant, *, **, *** Significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

carbohydrate accumulation had a small contribution to leaf osmotic adjustment potential. Their contribution to the Ψ_π did not change with the increase in NaCl concentration (9 to 14%), as the increase in leaf soluble carbohydrate content was proportional to the increase in leaf osmolality. Thus, other solutes appear to have decreased.

4. Discussion

Water status is highly sensitive to salinity and is, therefore, a dominant factor in determining plant responses to salt stress [1]. The results clearly showed that water relations of salt-tolerant genotypes were the same as salt-sensitive genotypes and, the genotype with the greatest osmotic adjustment was one of low salt tolerant (PaWS). There was no statistical difference for genotypes in the degree of ion accumulation and osmotic adjustment. Although the greatest change in total osmotic potential occurred in the genotype PaWS which also showed the greatest change in ion accumulation (K^+ and Na^+) (Table 1), the genotype showing the second greatest change in total osmotic adjustment (PvCS) had the least change in ion accumulation. In both PaWS and PvCS, the change in total osmotic potential was lower than that calculated for the ions, so other solutes must have decreased in concentrations in the salt-treated plants. This indicates that NaCl did not limit osmotic adjustment in salt-stressed plants and other organic solutes play an important part, independently, despite the higher energy costs for their synthesis [13].

The salt-induced soluble sugar accumulation had a small contribution to leaf osmotic potential (Table 3). The net increase in soluble carbohydrate fractions contributed about 14% of the measured decrease in leaf osmotic potential. Salinity increased carbohydrate content in leaves of *P. acutifolius* (PaWS and PaCT) (Table 2) but had a limited contribution to osmotic adjustment. Therefore, the hypothesis that assigns soluble carbohydrates a role in maintaining high turgor potential in leaves of *Phaseolus* species under prolonged stress can be dismissed. Despite the contrasting information found in the literature on the role of carbohydrates as osmolytes [34, 35], data presented in this work together with previous research [13, 23, 36] seem to indicate that the osmotic adjustment in *Phaseolus* species under salt stress is mostly dependent upon accumulation of inorganic ions. The important reductions in hexoses and sucrose in leaves of *P. vulgaris* (PvCS) (Table 2) proportional to the degree of salinization could be a consequence of the decrease in CO_2 assimilation (Figure 3) and might account for the impairment in plant growth and metabolism generally found in response to salt stress.

Although there were not genotypic differences in soluble carbohydrate contribution to the Ψ_π, the accumulation of

Table 3: Leaf water relations (MPa) of *Phaseolus* species after 20 days salt exposure[z].

Genotypes	Ψ_π	Ψ_π salt (mM NaCl)		$\Psi_{\pi s60} - \Psi_{\pi C}$	$\Psi_{\pi s90} - \Psi_{\pi C}$
	Control	60	90		
PvWT	−0.94 ± 0.13	−1.11 ± 0.11	−1.21 ± 0.12	0.18 ± 0.04	0.27 ± 0.04
PvCS	−0.88 ± 0.06	−1.13 ± 0.12	−1.22 ± 0.13	0.25 ± 0.05	0.34 ± 0.07
PaWS	−0.97 ± 0.12	−1.21 ± 0.11	−1.39 ± 0.15	0.20 ± 0.04	0.42 ± 0.06
PaCT	−0.87 ± 0.05	−1.10 ± 0.10	−1.18 ± 0.11	0.23 ± 0.03	0.31 ± 0.05
Na + K + Cl					
PvWT	−0.69 ± 0.07	−0.78 ± 0.11	−0.90 ± 0.10	0.09 ± 0.01	0.20 ± 0.05
PvCS	−0.63 ± 0.06	−0.69 ± 0.08	−0.79 ± 0.08	0.07 ± 0.02	0.16 ± 0.03
PaWS	−0.59 ± 0.05	−0.70 ± 0.07	−0.91 ± 0.12	0.11 ± 0.05	0.32 ± 0.09
PaCT	−0.50 ± 0.04	−0.63 ± 0.07	−0.76 ± 0.08	0.13 ± 0.05	0.26 ± 0.08
Glu + Fru + Suc					
PvWT	−0.09 ± 0.03	−0.06 ± 0.02	−0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
PvCS	−0.08 ± 0.02	−0.10 ± 0.04	−0.06 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
PaWS	−0.04 ± 0.01	−0.07 ± 0.03	−0.06 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
PaCT	−0.05 ± 0.01	−0.09 ± 0.04	−0.03 ± 0.01	0.04 ± 0.02	0.02 ± 0.01

[z]Data are for osmotic potential at full turgor (Ψ_π) of control and salt treatments, the degree of osmotic adjustment (($\Psi_{\pi s} - \Psi_{\pi C}$), as the difference of Ψ_π between the salt treatments and control plants), the contribution of Na^+, K^+, and Cl^- and glucose (Glu), Fructose (Fru) and Sucrose (Suc) to Ψ_π at 20 d. Values show means ± s.e. ($n = 6$).

sugars appears to be a common response in *P. acutifolius* (PaCT and PaWS) genotypes when grown under osmotic stress (Table 2). A similar finding in a comparison of rice varieties was reported by Cha-Um et al. [37]. They found that the total soluble sugar content in leaf and root tissues of salt-tolerant rice variety was higher than in the salt-sensitive variety, and that sugars enhance resistance to salt-induced osmotic stress in rice plants. Accumulated soluble sugars including glucose, fructose, and sucrose in the leaf tissues may function as osmoregulant solutes stabilizing photosynthetic pigments and maintaining electron transport functions during light reaction, and O_2 assimilation during dark reaction of photosynthesis. Sugars play a key role in the adaptive processes linked with NaCl-tolerance, such as Na^+ and Cl^- translocation and/or compartmentation, solute synthesis for growth, osmotic adjustment, and protein turn-over [38]. Sucrose has been shown to reduce oxygen activity of *Rubisco* during salt stress [38] and might be of primary importance in antioxidative mechanisms [14]. Further attention to determine if *P. acutifolius* has better osmoprotective functions or more efficient mechanisms to regulate photosynthetic rate parameters should be given.

There were significant differences between genotypes in their growth response to salinity. For salt-tolerant genotypes, RGR was reduced by salinity only in the first period (10–15 d) after salt treatment. After 15 d there was no significant difference in RGR between control and salt treatments. However, for salt-sensitive genotypes, the treatment differences in RGR were steady with time. Similar results were found by Rivelli et al. [39] for wheat grown at 150 mM NaCl for 30 d. The authors found that the greater effect on RGR occurred within the first 10 d of treatment, after which the difference between treatments largely disappeared. However, in an experiment with barley, the treatment differences in RGR were steady with time, over a 9-week period, RGR averaged 0.13, 0.09 and 0.09 for the 0, 100 and 175 mM NaCl treatments, respectively [40].

At the whole-plant level, decreases observed in RGR could be attributed to a photosynthetic response (ULR) and/or morphological changes (LAR), depending on the species [19]. Results confirm that decreases on RGR for salt-sensitive genotypes were related to ULR ($r^2 = 0.95$), indicating that the reduced growth in these species under high salinity was primarily as a result of a decline in leaf photosynthetic rate, as indicated by the lower stomatal conductance ($r^2 = 0.62$). These results support those reported by Romero and Marañón [17] and Bayuelo-Jiménez et al. [23], where the ULR was also found to be highly correlated with RGR for salt-stressed barley and beans, respectively.

In both salt-tolerant and salt-sensitive genotypes any decrease in SLA was consistently associated with a decline in LAR ($r^2 = 0.94$), and consequently in RGR (Figure 2). Growth of salt-tolerant genotypes was affected by a reduced leaf area expansion (smaller and thicker leaves) rather than impairment on CO_2 assimilatory capacity. In salt-sensitive genotypes, however the lower SLA may reflect an overloading of the leaves by inorganic (Cl^-; $r^2 = 0.85$ to $r^2 = 0.98$) solutes, which allow osmotic adjustment but decreased photosynthetic return per unit leaf mass.

Specific leaf area (SLA) is a variable associated with a number of functional aspects of plant physiology, including gas exchange and relative growth rate [19]. Generally, evidence shows that salinity increases the leaf lamina thickness, due to an increase in mesophyll cell size or number of layers [41, 42]. Such salt-induced succulence could lower the resistance to CO_2 uptake and thus increase photosynthetic rates by increasing the amount of internal leaf surface area across which gaseous exchange can occur per unit of

leaf area [41]. We suggest that the lower SLA of the salt-tolerant genotypes may reflect an increase in mesophyll thickness and the internal surface area for CO_2 absorption, which probably compensates for any stomatal assimilation limitation. Although the role of stomatal and mesophyll resistance in controlling the CO_2 diffusion resistance has yet to be proven in salt-tolerant *Phaseolus* species, the proven effects of changes in salt-sensitive *P. vulgaris* is restricted to stomatal conductance [41].

The decrease in conductance in salt-stressed plants could be due to chemical signals coming from the roots or reduced shoot water content [43]. Our data indicate that decreased conductance was not due to leaf water deficit since the calculated turgor was not reduced by salinity, whereas conductance was (Figure 4). This suggests hormonal control originating from the roots [43]. According to Rivelly et al. [39], values of carbon isotope discrimination (Δ) measured on expanding leaf tissue of wheat genotypes was substantially lower for salt-stressed plants than for control plants. This indicates that the effects of salinity on stomatal conductance were greater than effects on photosynthetic capacity. Thus, the reduction of the photosynthetic capacity of salt-tolerant *Phaseolus* species may not reflect an apparent damage to photochemistry and chlorophyll concentrations; however, more information is necessary for conclusion on this point.

It has also been proposed that the reduction of A_n in response to salinity is due to an increase on Na^+ and Cl^- leaf contents [18, 35, 36]. However, other authors reported associated reductions in A_n and g_s with K^+ deficiency [44]. Because salt stress impairs K^+ uptake of plants, it has been suggested that K^+ deficiency might be a contributing factor to salt-induced oxidative stress and related cell damage. Due to impairment in: (1) stomata regulation, (2) conversion of light energy into chemical energy, and (3) phloem transport of photosynthates from leaves into sink organs, photosynthetic CO_2 fixation is limited [44]. It is therefore possible that in salt-sensitive *Phaseolus* species potassium deficiency combined with salt stress induced a reduction in CO_2 photo assimilation and stomata closure (Figure 3).

The results also showed that high foliar concentrations of Cl^- were related with reduced A_n in PaWS ($r^2 = 0.66$) and PvCS ($r^2 = 0.83$) genotypes. It is interesting that leaf Cl^- concentrations of salt-tolerant PvWT and PaCT remained relatively high but did not inhibit photosynthesis (Figure 3). This seems to exclude the possibility that more intense inhibition of leaf growth expansion in salt stressed of these species was caused by Cl^- toxicity on leaves and/or photosynthesis inhibition. Cl^- is an important inorganic ion and might also play key roles in osmotic adjustment. For example, Shabala et al. [45] suggested a role of the hyperosmolarity induced influx of K^+ and Cl^- in plant (e.g., bean) cells that could be sufficient for osmotic adjustment without additional accumulation of organic solutes. Under conditions of saline stress, excess concentration of Cl^- occurs in plants, and the Cl^- channel might be involved in change of cellular Cl^- content for osmotic adjustment.

Osmotic adjustment and turgor maintenance were achieved by inorganic ions uptake, but an imbalance of essential nutrients may also be a factor contributing to the salt-induced decrease in leaf function and consequently in plant growth [1]. Excess NaCl in the external solution induced a reduction in the vegetative growth of salt-sensitive genotypes, which correlated with the accumulation of Cl^- in plant tissues (Table 1). The addition of salt ions (Na^+ and Cl^-) to the nutrient solution was reflected in higher absorption rates of these ions by the plant. However, chloride absorption was higher for salt-sensitive PvCS than for all other species, probably due to the higher proportion of young root zones [25].

Plant growth can be stimulated by low concentrations of sodium, mainly as a result of the effects of Na^+ on cell expansion and cell water balance [7]. Na^+ transport from root to shoot seemed to be more strongly inhibited than absorption, as deduced from the higher concentrations of salt ions measured in roots than in foliar tissues of all *Phaseolus* species (Table 1). On the other hand, the toxic effects of salt ions and/or a deficiency of particular nutrients may inhibit plant growth [4]. Chloride, the other salt ion, has a high mobility within the plant and affects processes related to charge compensation and osmoregulation [1]. For salt-sensitive genotypes, a greater concentration of Cl^- in leaves was associated with reduced plant growth (Figure 2).

A decrease in potassium accumulation in salt-stressed plants appears to be one of the most widespread responses associated with reduced growth [17, 44]. Salinity affected the potassium accumulation in the vegetative phase, probably by significantly reducing the absorption of potassium in roots (Table 1). The K^+ concentration fell continuously in roots and stems of salt-stressed species, while the leaves had similar concentrations to those in control plants at the medium salt stress levels, suggesting a compensation over time, probably by translocation of K^+ from roots and stems to leaves [17], a sustained acquisition despite appreciable overall Na^+ uptake [23], and/or a high K^+ selectivity and/or K^+/Na^+ exchange across the plasmalemma of the root epidermis [6, 9, 10]. The ability to withdraw Na^+ and to retranslocate K^+ seems crucial for salt tolerance [10, 11]. Therefore, the maintenance of higher leaf K^+ concentrations in PvWT could be an important mechanisms underlying superior salt tolerance reported in *P. filiformis* [23] and barley (*H. vulgare* L.) [10].

Legumes are a key component of sustainable agriculture and can offer many economic and environmental benefits if grown more widely in crop rotations because of their ability to fix nitrogen in the root nodules in a symbiotic interaction with soil rhizobia. Due to their capacity to grow on nitrogen-poor soils, they can be efficiently used for improving saline soil fertility and help to reintroduce agriculture to these lands [46, 47]. However, in legumes, salt stress imposes a significant limitation of productivity related to the adverse effects on the growth of the host plants, the root nodule bacteria, symbiotic development, and the nitrogen fixation capacity [46].

Possible approaches to improve productivity under saline stress conditions require a better understanding of the physiological and molecular mechanisms involved in the response to salt stress. These mechanisms include (1) exclusion of Na^+ and Cl^- from plant tissue, (2) inclusion of these ions in inert compartments or tissues, and/or (3) some means

of osmotic adjustment with solutes that are compatible with the metabolic machinery of the cell [46]. Conventional plant breeding based on yield in target environments has increased production; however, physiologically based approaches utilizing molecular tools to identify key genes or provide molecular markers have the potential to take it further [47]. Accurate and selective phenotyping will enable to best use of mechanistic molecular understanding of plant responses to salinity, and mechanisms of adaptation [1].

5. Conclusions

From the present study, we conclude that salt-induced growth reductions in salt-sensitive *Phaseolus* species during vegetative growth are due to a decrease in the specific activity of the leaves (ULR). In contrast, a reduced leaf area expansion per unit of plant biomass (LAR), primarily caused by a decrease in SLA, played an important role in determining RGR of salt-tolerant species. The lower ULR of salt-sensitive species may be a result of decreased photosynthesis due to a decreased leaf water vapor conductance. Leaf water relations, however, seem unlikely to be a growth-limiting factor in *Phaseolus* species. There was little difference between genotypes in the effect of salinity on water relations, as indicated by the estimated turgor. Osmotic adjustment occurred in all *Phaseolus* species, with one of the low salt tolerance genotypes having the greatest osmotic adjustment. A higher level of soluble carbohydrates was found in salt-tolerant species. However, the salt-induced soluble sugar accumulation does not play a significant role in defense against osmotic stress conditions. Salt-sensitive *Phaseolus* species are Na^+ excluders and maintained turgor-driven extension growth by accumulating Cl^- (osmotic adjustment), but subsequent weight gain reductions suggest that this led to ion toxicity.

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Research Article

Genetic Transformation of Common Bean (*Phaseolus vulgaris* L.) with the *Gus* Color Marker, the *Bar* Herbicide Resistance, and the Barley (*Hordeum vulgare*) *HVA1* Drought Tolerance Genes

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Five common bean (*Phaseolus vulgaris* L.) varieties including "Condor," "Matterhorn," "Sedona," "Olathe," and "Montcalm" were genetically transformed via the Biolistic bombardment of the apical shoot meristem primordium. Transgenes included *gus* color marker which visually confirmed transgenic events, the *bar* herbicide resistance selectable marker used for *in vitro* selection of transgenic cultures and which confirmed Liberty herbicide resistant plants, and the barley (*Hordeum vulgare*) late embryogenesis abundant protein (*HVA1*) which conferred drought tolerance with a corresponding increase in root length of transgenic plants. Research presented here might assist in production of better *P. vulgaris* germplasm.

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is a very important source of vegetable protein, especially in those regions of the world in which animal proteins are scarce. Common bean provides 22% of the total protein requirement worldwide [1]. Conventional breeding has contributed significantly to the trait improvement of *P. vulgaris*. However, breeding cannot add certain genes that do not exist naturally in the *P. vulgaris* gene pool. Due to this limitation of plant breeding, new trait improvement approaches such as interspecific horizontal gene transfer via genetic engineering need to be utilized in order to complement the limitations encountered by conventional breeding of this crop [2, 3].

Mostly, *Agrobacterium*-mediated transformation and the gene gun microprojectiles bombardment method have been used for genetic transformation of *P. vulgaris.* However, neither system has shown as high as those seen in genetic transformation of cereals [4]. Researchers have unsuccessfully attempted to transform *P. vulgaris* protoplast, either via polyethylene glycol or electroporation [5]. A relatively advanced *Agrobacterium*-mediated transformation of *P. vulgaris* has been reported on the use of sonication and vacuum infiltration for transfer of a group of 3 LEA (late embryogenesis abundant protein) genes from *Brassica napus* [6]. Although the transformation efficiency using this system was low, transgenic plants exhibited a high growth rate under salt and water stress. A recent report [7] on transformation of *P. vulgaris* varieties Mwitemania and Rose coco using the *gus* color marker gene reveals the importance of specificity of *Agrobacterium* strains in expression of *gus* gene in *P. vulgaris*. For example, infecting of *P. vulgaris* explants with EHA 105 (pCAMBIA 1201) or EHA 105 (pCAMBIA 1301) resulted in blue GUS coloration; however, it did not show the GUS expression when the explants were infected with LBA 4404 (pBI 121) *Agrobacterium* strain.

Using Biolistic bombardment of a construct containing the *bar* gene, Aragão et al. [3] developed transgenic *P. vulgaris* which conferred resistance to glufosinate ammonium, the active ingredient of Liberty herbicide (Aventis, Strasbourg, France), at concentrations of 500 g ha^{-1} in greenhouses and 400 g ha^{-1} in the field. *P. vulgaris* was also genetically engineered by Bonfim et al. [8] using RNAi-hairpin construct to silence the AC1 region of the viral genome of Bean Golden Mosaic Gemini Virus (BGMGV). However, out of 2,706 plants, only 18 putative transgenic lines were obtained.

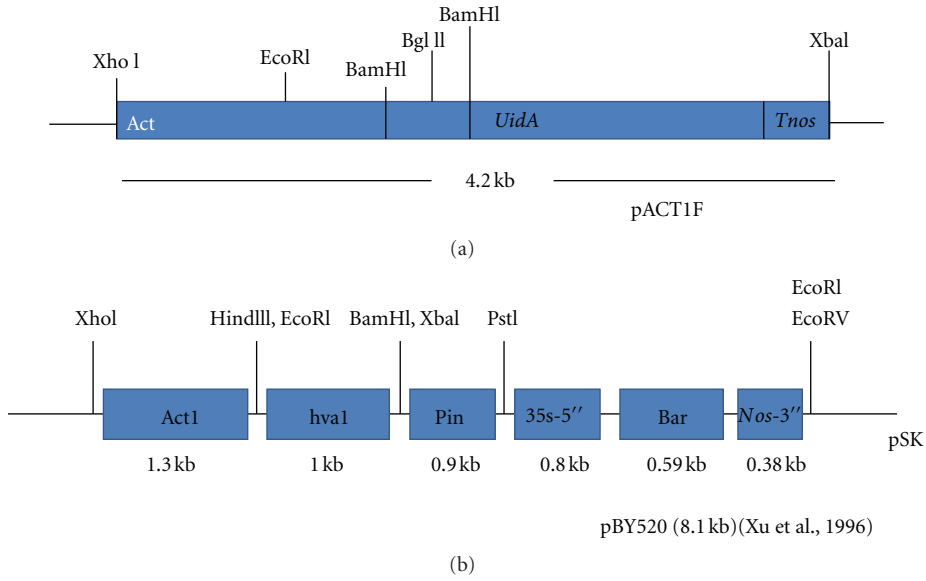


Figure 1: Plasmid constructs. (a) Linear map of pACT1F plasmid vector. Rice actin promoter (Act1), *gus* gene (*uidA*), and nopaline synthase terminator (*Tnos*); (b) linear map of pBY520 plasmid vector. Rice actin promoter (Act1) and potato protease inhibitor II (Pin II-3′) terminator, Barley or *Hordeum vulgare* (*HVA1*) LEA 3 gene, Cauliflower Mosaic Virus 35S promoter, *bar* gene and nopaline synthase terminator (*Nos*-3′).

Of the 18 putative transgenic plants, only one plant exhibited resistance to the virus. Field trials of the progenies of the single transgenic plant showed resistance to this virus [9]. Vianna et al. [10] developed an approach of transferring the transgene assembly as fragment pieces of DNA, as opposed to the entire plasmid into *P. vulgaris*. A protocol was published [11] on a relatively efficient genetic transformation of *P. vulgaris*. Due to the "troublesome" nature of *P. vulgaris* genetic transformation, an article describes a method called "transgenic composite" of *P. vulgaris* via the use of *Agrobacterium rhizogenes* transformation of derooted seedlings [12].

The efficiency of genetic engineering of *P. vulgaris* has remained a challenge. A relatively recent report explains the effect of *in vitro* conditions on indirect organogenesis (multiple shoots from meristem and cotyledon-derived callus regeneration) for production of an average of 0.5 shoot per callus clump. Indirect regeneration of different genotypes of *P. vulgaris* was also reported [13]. Kwapata et al. [14] cite that an *in vitro* culture of a single apical shoot meristem primordium could produce as many as 20 multiple shoots, which is a relatively higher number as compared to the work previously presented. However, this *in vitro* regeneration efficiency is still very low when compared to the desired 100s regenerated from the *in vitro* cultures of each apical shoot meristem primordia of cereal crops [4].

Genetic transformation of *P. vulgaris* can improve the biotic and abiotic stress tolerance. Biotic stress factors such as diseases result in *P. vulgaris* yield loss. Brazil just announced [15] the commercial use of golden mosaic virus resistant *P. vulgaris* that was developed via RNA interference by blocking the replication of the virus gene [8]. This is indeed a major step in the acceptance of biosafety of transgenic *P. vulgaris*. Also, researchers from Denmark recently reported cloning of the bean common mosaic virus (BCMV) gene and its application for development of BCMV resistance [16].

Biotic stresses, including drought cause plants to lose cellular turgidity, followed by the aggregation and misfolding of proteins and yield losses [17]. A major group of abiotic stress tolerance genes coding for the late embryogenesis proteins include a class of heat shock proteins (Hsp) that are extremely hydrophilic and resilient towards heat, such that they do not coagulate at boiling temperatures. The LEA proteins play a role in water binding, ion sequestration, and macromolecule and membrane stabilization [18]. In the research presented here, the barley *HVA1* [19] gene was transferred into *P. vulgaris,* as this gene encodes a type III LEA protein. The Barley *HVA1* gene has previously been transferred to rice [20], wheat [21, 22], sugarcane [23], creeping bentgrass [24], mulberry [25], and oat [26, 27]. In all cases, plants developed tolerance to abiotic stresses such as drought and/or salt. Here we report the transfer of Barley *HVA1* gene to different varieties of *P. vulgaris* and report the development of drought tolerance of transgenic plants at greenhouse level.

2. Materials and Methods

2.1. Plasmids and Explant. Two different plasmid vectors were used in this research (Figure 1). Plasmids used included (a) pACT1F harboring the *gus* gene and (b) pBY520 harboring the *HVA1* and the *bar* gene, which confers drought tolerance and Liberty herbicide (glufosinate ammonium) resistance, respectively.

Explant preparation: the explant used to standardize the genetic transformation was *P. vulgaris* var. "Sedona."

Dry seeds were rinsed in tap water for 1 min, then rinsed three times with distilled water, soaked in 75% ethanol for 4 min, and again rinsed three times with distilled water. Then, the seeds were soaked in 20% commercial Clorox while steering for 15 min.

Seed coats of the surface-sterilized seeds were removed, and meristems were dissected under a light microscope under a laminar flow hood. The meristem dissection took place by removal of the cotyledons and the hypocotyls, leaving the meristem as an intact explant.

The meristem explants were cultured in Murashige and Skoog (MS) [28] medium containing 2.5 mg^{-1} benzyl adenine (BA; Sigma-Aldrich, Inc. Steinheim, Germany) and 0.1 mg^{-1} indole acetic acid (IAA; Sigma-Aldrich, Inc. Steinheim, Germany). Cultures were maintained under *in vitro* conditions and in a dark chamber for 5–7 days or until the explants grew to about 5–7 mm long. Then, 10 of the elongated apical meristems were placed in a circle in a Petri dish on top of MS medium, bombarded with gene constructs using the Biolistic gene via the helium particle delivery model PDS-1000 (DuPont, Wilmington, DE).

The pACT1-F construct containing the *gus* gene was coated onto 50 $\mu g\,L^{-1}$ of 10 μm tungsten particles with 2.5 M calcium chloride and 0.1 M spermidine suspended in a solution of 1 : 1 (v/v) of 75% ethanol and 50% glycerol. The coated plasmid DNA was bombarded into the explants using three levels of pressure (500, 1000, or 1100 psi), plasmid concentrations of 1.5 μg or 3.0 μg, and with three levels of bombardment frequencies (1, 2 or 3 time). A total of 10 apical meristems were used for each bombardment condition.

The bombarded shoot meristems were transferred to regeneration medium [14] and kept under *in vitro* condition at room temperature with 16 h photoperiod and light intensity of 45–70 umol $m^{-2}s^{-1}$.

The bombarded shoot meristems were histologically stained to visualize the *gus* gene expression, and three longitudinal hand-cross-sections of each bombarded shoot meristem were made to identify the bombardment criteria that lead to expression of *gus* gene in relative location of *P. vulgaris* meristem subepidermal layer. Mean of transient transformation efficiencies (number of meristems showing blue spots) was used as preliminary data to identify the most acceptable criteria of bombardment (Table 1).

The most effective criteria were then used for stable transformation of the five varieties of *P. vulgaris*. The GUS histological assay bombarded versus control wild-type meristems included histochemical staining with 5-bromo-4-chloro-3-indoyl-β-D-glucuronicacid salt (X-gluc). Samples were dipped into GUS substrate buffer, according to published records [29], and incubated at 37°C for 24 hours. The tissue samples were washed with 100 percent ethanol to remove other colorations.

The statistical design used in this portion of research was a completely randomized design (CRD). An Analysis of Variance (ANOVA) was used to test the statistical significance at an alpha level of 0.001. Standard deviations were used to compare variability.

2.2. Stable Genetic Transformation. Stable genetic transformation of *P. vulgaris* was performed using the Biolistic delivery for bombardment of a 1 : 1 ratio mixture of the two plasmids into the apical shoot subepidermal cell layer area using the ideal bombardment criteria (Table 1). The bombarded explants were cultured in regeneration media [14] without the use of any chemical selections for 24 hours.

The selection of stable transgenic plants was based on the use of *gus* color marker gene and 4 $mg\,L^{-1}$ of glufosinate ammonium selection for the *bar* herbicide resistance marker gene. The *in vitro* regeneration of putatively transgenic *P. vulgaris* explants followed a previous report [14].

2.3. Confirmation of Transgene Integration and Expression

2.3.1. Polymerase Chain Reaction (PCR). Polymerase chain reaction (PCR) was used for detection of integration of *bar* and *HVA1 trans*genes in four generations (T_0–T_3) of plants that were putatively transformed with Biolistic gun. The primers used were (1) *bar* F, 5′-ATG AGC CCA GAA CGA CG-3′ (forward primer); *bar* R, 5′-TCA CCT CCA ACC AGA ACC AG-3′ (reverse primer); (2) *HVA1* F, 5′-TGG CCT CCA ACC AGA ACC AG-3′ (forward primer); *HVA1* R, 5′-ACG ACT AAA GGA ACG GAA AT-3′ (reverse primer).

2.3.2. Southern Blot Hybridization. The Southern blot hybridization analysis was conducted to determine the stability of transformation and to determine the copy numbers of the *bar* and *HVA1 trans*genes. The DIG High Prime DNA Labeling and Detection Starter Kit (Roche Co., Cat. No. 1 585 614) was used as per manufacturer's instructions. Transgenic and control wild-type nontransgenic genomic DNA was isolated using methods described [30]. The DIG-labeled probes for *bar* and *HVA1* were synthesized using primers for specific genes as described previously. Those transgenic plants that integrated 1-2 copies of transgenes were kept for further studies.

2.3.3. Northern Blot Hybridization. Northern blot analysis was conducted using the DIG-labeled Northern Starter Kit (Roche Co., Cat. No. 12039672910). Total RNA from the leaves of transgenic and the control wild-type nontransgenic plants was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO) as per manufacturer's instructions. A total of 30 μg of RNA per sample was loaded onto a 1.2% (m/v) agarose-formaldehyde denaturing gel as described [31] and transferred to a Hybond-N+ membrane (Amersham-Pharmacia Biotech) and fixed with a UV crosslinker (Stratalinker UV Crosslinker 1800, Stratagene, CA). The RNA or DNA DIG-labeled probe, containing the coding region of the gene of interest, was used for detection of transcripts.

2.4. Biological Activity Tests

2.4.1. Herbicide Resistance Assay. Following a glufosinate ammonium *in vitro* culture kill curve studies (data not shown), an optimum 4 $mg\,L^{-1}$ of glufosinate ammonium

was used in the *in vitro* culture of putatively transgenic shoot regeneration and rooting media.

Different concentrations of Liberty herbicide (50, 100, 150, 250, or 350 mg L^{-1}) were used to find the ideal foliar spray concentration of trifoliate transgenic plants.

In vitro germination of progeny seeds in MS medium [28] containing 4 mg L^{-1} of glufosinate ammonium was used to indentify segregation ratio of the *bar* transgene in transgenic progenies.

2.4.2. Drought Tolerance Test. The *HVA1* transgenic and wild-type control seeds were collected, and seedlings were grown in 15 cm clay pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX) in a growth chamber for three weeks or until trifoliate leaves appeared. Plants were watered daily for 21 days, after which moisture was withheld for 21 days. Then, water was applied to plants continuously for up to 14 days, and the percentage of plants recovered was recorded. Also, percent plant leaf abscission was used as an indirect measure of degree of plant wilting. In reality, the number of green leaves on plants after 21 days of moisture withdrawal was used to find percent plant leaf abscission.

3. Results and Discussions

3.1. Explant. Our results show that the apical shoot meristem primordium might be a good explant for genetic transformation of common beans. The apical shoot meristem in *P. vulgaris* is an undifferentiated meristematic tissue in a small and relatively round shape, which is composed of different cell layers. The top layer or the "Epidermal Cell Layer" divides horizontally and will not differentiate. The layer beneath the Epidermal Cell layer is the "Subepidermal Cell Layer" (also called the primordial cell layer or stem cell layer) normally divides indefinitely and differentiates into gametes resulting into fertile plants. Therefore, it is the Subepidermal Cell Layer that needs to be targeted via the Biolistic gun for genetic transformation. Using the gus color marker gene, the researchers of this report tried to standardize the Biolistic delivery bombardment to hit this layer.

3.2. Transient Expression of the Gus Marker Gene. Bombarding the explants twice at the approximate distance of 4 cm between the gun barrel and target explants, using a pressure setting of 1100 psi, with a concentration of 1.5 μg of plasmid DNA per bombardment yielded the highest GUS activity efficiency of 8.4% (Table 1). Mean transient transformation was calculated by counting the mean of number of bombarded meristems that showed blue spots.

The transient transformation frequency of the GUS expression is shown in Figure 2. The number of clear blue spots was seen 15 days after bombardment.

3.3. Stable Transformation. PCR was performed for all *bar* and HVA1 transgenes used, among which results are only shown for integration of HVA1 transgene in all four *P. vulgaris* cultivars (Figure 3(a)). Southern blot hybridizations were performed in multiple samples of PCR-positive plants, among which data are only shown for integration of HVA1 gene in different *P. vulgaris* cultivars. After Southern blot hybridization analysis, transgenic plants that showed the integration of at most two copies of transgenes (e.g., see Figure 3(b)) were kept for transcription analysis. Transcription analysis via RT-PCR showed that HVA1 has transcribed in all transgenic plants. However RNA blotting confirmed that only certain transgenic plants sufficiently transcribed their transgenes (e.g., see Figure 3(c)). This is because RT-PCR is much more sensitive than the RNA blotting.

Table 1: Transient expression of GUS using different gene gun pressure (psi), DNA plasmid concentration and bombardment frequency for optimizing the Biolistic bombardment conditions for the pCATIF containing the *gus* gene.

Bombardment pressure (psi)	Concentration of plasmid DNA (μg)	Bombardment frequency	Mean transformation percent
500	1.5	1	0.1 ± 0.04
500	1.5	2	0.2 ± 0.10
500	1.5	3	0.4 ± 0.30
500	3	1	0.1 ± 0.04
500	3	2	0.6 ± 0.32
500	3	3	0.7 ± 0.32
1000	1.5	1	2.9 ± 0.67
1000	1.5	2	3.9 ± 1.4
1000	1.5	3	5.1 ± 1.2
1000	3	1	5.6 ± 1.0
1000	3	2	8.1 ± 0.3
1000	3	3	7.4 ± 1.0
1100	1.5	1	7.2 ± 0.70
1100	1.5	2	8.4 ± 0.74
1100	1.5	3	8.2 ± 0.50
1100	3	1	7.5 ± 0.69
1100	3	2	4.8 ± 0.93
1100	3	3	3.3 ± 0.92

The GUS bioassay was a method of selecting the transgenic shootlets. All Southern blot-positive progenies of *P. vulgaris* varieties ("Matterhorn," "Condor," "Sedona," "Olathe," and "Montcalm") showed GUS expression. Figure 4 represents expression of GUS protein in seeds and pods of T3 of "Matterhorn."

Because glufosinate ammonium was included in the *in vitro* cultures of all putatively transgenic shoots, roots and plantlets, all transgenic plant progenies were resistant to 150 mg L^{-1} of Liberty herbicide (Figure 5). Lower concentrations did not kill wild-type control nontransgenic plants, and higher concentrations killed transgenic plants as well as their wild-type control non-transgenic counterparts.

Most drought tolerant HVA1 transgenic plants were "Sedona" and "Matterhorn" which persisted for 21 days without irrigation. They showed symptoms of drought stress but recovered only after three days when moisture application resumed. The wild-type control plants died or showed severe symptoms of drought stress, with most of their leaves

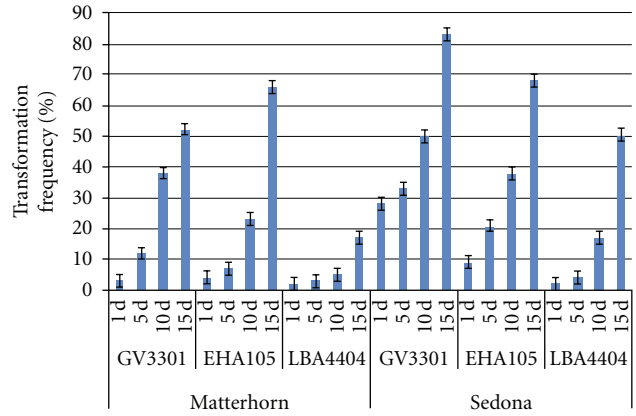


Figure 2: Percent transient expression of GUS at different number of days after bombardment.

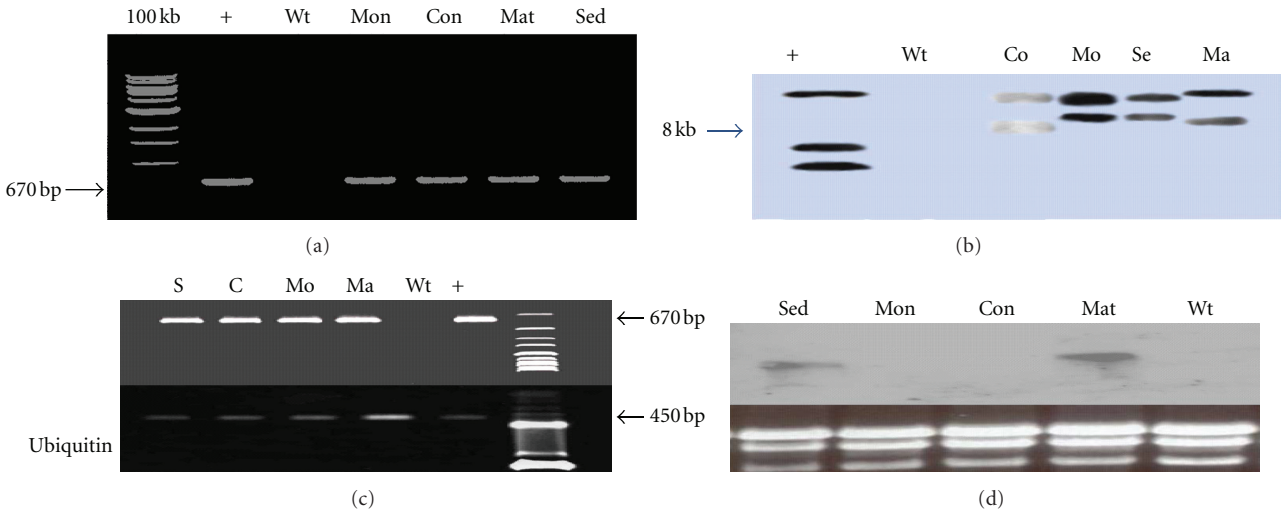


Figure 3: Molecular analysis confirming the integration and transcription of HVA1 transgene in plants. (a) PCR results of T3 transgenic plants. The expected band size is 670 bp; (b) Southern blot hybridization showing integration of *HVA1* gene digested with BamH1. The results indicate that there are two copies of transgene in all varieties. (c) RT-PCR of *HVA1* expression for T2 transgenic plants. Like in PCR, here the expected band size is 670 bp for *HVA1*. Below the RT-PCR is the cDNA loading control showing the expression of ubiquitin with the expected band size of 450 bp. (d) Northern blot analysis also confirmed the transcription of *HVA1* gene in Sedona and Matterhorn. Wt: wild type shows no transgene integration; C. RNA transcription analysis of *HVA1* gene in T3 transgenic plants. Mat: "Matterhorn" and Sed: "Sedona" showed some expression. The remaining lanes, Wt: wild type, Mon: "Montcalm" and Con: "Condor" showed no transcriptions.

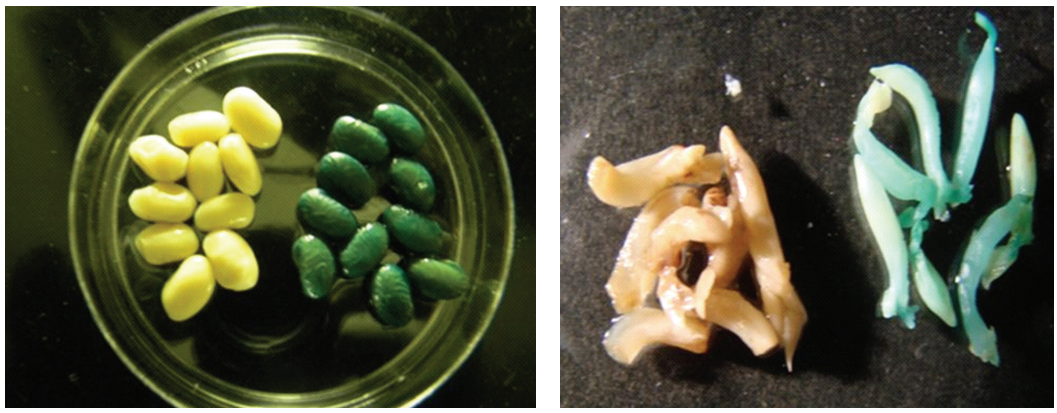
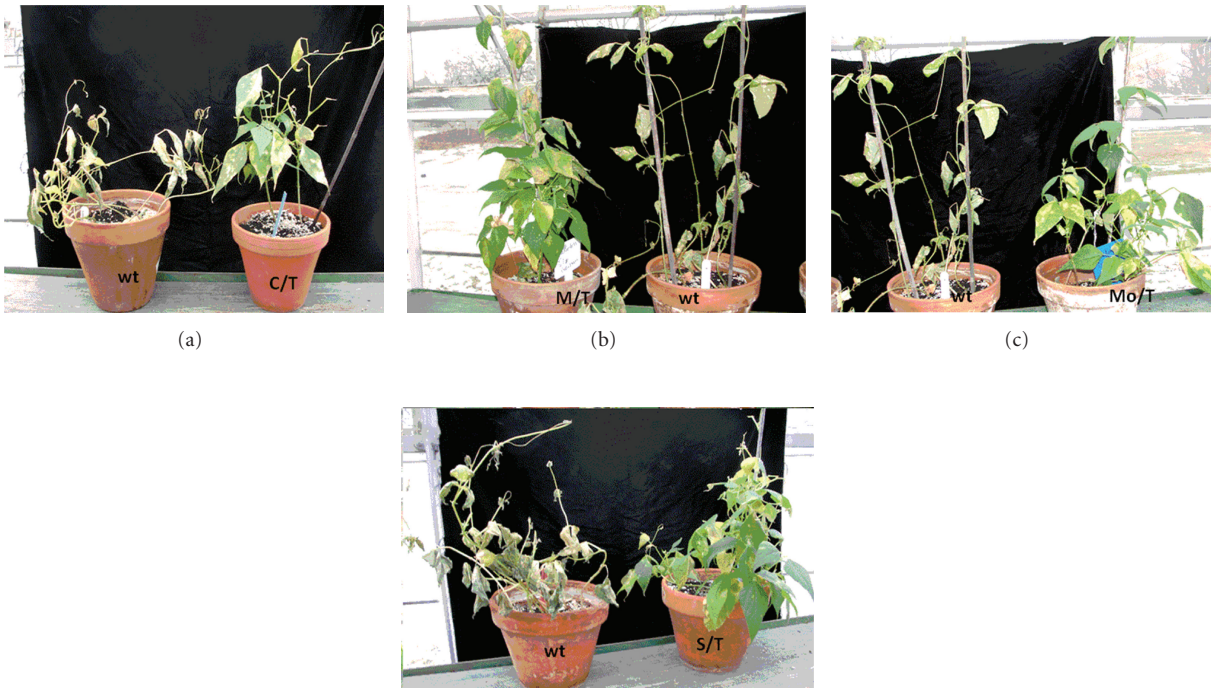


Figure 4: GUS biological activity shown after histochemical assays in pods and seeds of T3 "Matterhorn."



(a) (b) (c)

(d)

Figure 5: Although not completely resistant, the trifoliate stage of the third generation transgenic (T2) plants that had transcribed *bar* gene showed more resistance to foliar spray of 150 mg L^{-1} Liberty herbicide than their wild-type control non-transgenic counterpart plants. "Condor" (a), "Matterhorn" (b), "Montcalm" (c), and "Sedona" (d). "Matterhorn" seems to be more resistant to the herbicide.

(a) (b) (c)

(d)

Figure 6: Drought tolerance assays. (a) "Matterhorn" plants before drought induction; (b) after 21 days of continuous water withholding; (c) "Matterhorn" drought recovered plants after water reapplication; 1: control non-transgenic plant that was watered throughout the experiment; 2: "Matterhorn" transgenic plant after 21 days of no-irrigation, 3: wild-type non-transgenic plant after 21 days of no-irrigation; (d) root growth in plants after 21 days of drought stress. 1: Control non-transgenic plant roots; these were watered daily, 2: transgenic plant roots after 21 days of no-irrigation, and 3: wild-type non-transgenic plant roots after 21 days of no-irrigation.

being wilted and dehisced (e.g., see Figure 6(b)). The survival rate of control wild-type non-transgenic "Sedona" plants after 21 days of drought was 13.3% and for its HVA1 transgenic plants of the same variety was 33.3%. In case of "Matterhorn," the survival rate of control wild-type non-transgenic plants was 20%, and its transgenic counterpart of the same variety was 53.3%. Withdrawal of irrigation for more than 21 days resulted in the death of both control wild-type non-transgenic and HVA1 transgenic plants of "Sedona" and "Matterhorn" varieties.

The percent leaf wilting of transgenic "Sedona" plants was 78% and for its wild type was 91%. In the case of "Matterhorn," percent leaf wilting of transgenic plants of "Matterhorn" variety was 72% as compared to the wild-type control non-transgenic plants which were 88%.

Over all, the root growth of HVA1 transgenic plants with least percent wilting was more robust than wild-type plants under stress, but less developed than wild type plants under a normal moisture regime (e.g., see Figure 6(c)).

In a preliminary experiment, the average root length measurement after 21 days of water withdraw for "Sedona" HVA1 transgenic plants was 15 cm and for wild-type plants was 11 cm. For "Matterhorn" variety was 72% as compared to the wild-type control non-transgenic plants which was 88%. In contrast, for control wild type plants under normal irrigation without water withhold, the average root length was 28 cm.

The researchers of this paper exposed transgenic plants transcribing the HVA1 gene to drought prior to testing of plants for drought tolerance. The promoter deriving the HVA1 in this work is rice actin 1 promoter which is known to be a constitutive promoter. Transgenic plants might have shown more drought tolerance should the promoter used was an inducible one, such as *Arabidopsis* rd29 promoter [32].

4. Conclusions

GUS assay was essential to identify the relative location of the subepidermal area of explants as the target for Biolistic bombardment.

All plants transformed with the *bar* Liberty herbicide resistance gene showed stable expression of this gene because of continuous *in vitro* culture selections of explants, shootlets, and plantlets in media containing the active ingredient of this herbicide.

Our studies of transgenic *P. vulgaris* that expresses barley *HVA1* transgene agree with an earlier report [33] in which "Matterhorn" possesses a genotypic advantage over "Sedona" in terms of naturally tolerating drought.

The expression of barley *HVA1* gene in *P. vulgaris* resulting in drought tolerance agrees with results obtained from transfer of this gene into other crops and their tolerance to drought and/or salt [20–27].

Further studies are needed to locate the precise location of the subepidermal cell layer, possibly via the use of GUS monoclonal antibody followed by laser microscopy because GUS color easily diffuses from cell to cell.

Further studies are also needed to test *HVA1* transgenic *P. vulgaris* at the field level. The research presented here and the genes transferred into common bean varieties might improve the yield and economy of this important crop.

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Research Article

Role of Pigeonpea Cultivation on Soil Fertility and Farming System Sustainability in Ghana

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The productivity of the smallholder farming system in Ghana is under threat due to soil fertility decline. Mineral fertilizer is sparingly being used by smallholder farmers because of prohibitive cost. Grain legumes such as pigeonpea can play a complementary or alternative role as a source of organic fertilizer due to its ability to enhance soil fertility. Despite its importance, the potential of pigeonpea as a soil fertility improvement crop has not been exploited to any appreciable extent and the amount of land cultivated to pigeonpea in Ghana is vey negligible. This paper synthesizes recent studies that have been carried out on pigeonpea in Ghana and discusses the role of pigeonpea cultivation in soil fertility management and its implication for farming system sustainability. The paper shows that recent field studies conducted in both the semi-deciduous forest and the forest/savanna transitional agroecological zones of Ghana indicate that pigeonpea/maize rotations can increase maize yield by 75–200%. Barrier to widespread adoption of pigeonpea include land tenure, market, and accessibility to early maturing and high yielding varieties. The paper concludes among other things that in order to promote the cultivation of pigeonpea in Ghana, there is the need to introduce varieties that combine early maturity with high yields and other desirable traits based on farmers preferences.

1. Introduction

Agricultural productivity in the smallholder farming systems in Ghana is under threat due to declining soil fertility. In the past, smallholder farmers in Ghana relied on the extended bush fallow system for maintaining the productivity of their farmlands [1]. This system allowed restoration of phosphorus (P) and nitrogen (N), the most limiting nutrients. However, over the years, the population growth-induced scarcity of suitable farmland has led to the shortening of the fallow period making it difficult to manage soil fertility in smallholder farming systems. The problem is compounded by the increasing cost of inputs at the farm level due to structural adjustment programmes that have removed subsidies and increased supply costs due to the deterioration conditions of rural infrastructure [2]. For instance, in 2002, whereas a metric tonne of urea cost about US$90 FOB (free on board) in Europe [3], the same quantity cost a Ghanaian farmer about US$308 at the farm level [4].

Most farmers, especially the smallholder farmers, do not have access to formal credit and therefore cannot afford to buy mineral fertilizers even when it has been demonstrated to be profitable [5]. Furthermore, due to the unpredictability of rainfall in some areas, farmers consider fertilizer application as risky.

In smallholder farming systems in Ghana, legumes can play a complementary or alternative role as source of organic fertilizer. Research in many parts of Sub-Saharan Africa including Ghana has shown that legumes have the potential to sustain soil fertility in smallholder farming systems [6–8]. Pigeonpea (*Cajanus cajan* (L.) Millsp.) has been found to have a great potential in this respect because of its ability to recycle nutrients and tolerate wide environmental conditions and low soil fertility [9]. Pigeonpea is nutritionally well balanced and is an excellent source of proteins (20–30%) [7]. In addition to proteins, pigeonpea provides carbohydrates and high levels of vitamins A and C.

Despite the importance of pigeonpea in terms of nutrition and soil fertility improvement, the crop has not been promoted to any appreciable extent by the national agricultural research and extension system in Ghana. In the forest/savannah transitional zone where the crop is widely cultivated, pigeonpea is considered as women's crop and therefore minor due to men's dominion over women in household decision-making process. Furthermore the overemphasis on maize in the current production systems as the major food security and cash crop in the forest/savanna transitional zone has relegated pigeonpea in particular and legumes in general as a minor crop.

However, recent studies in the forest/savannah transitional agroecological zone as well as the semideciduous forest zone of Ghana have demonstrated that when integrated in the cropping system as a form of rotation, pigeonpea has the potential of improving soil fertility. This paper discusses the role of pigeonpea cultivation in soil fertility management and its implication for farming system sustainability and food security.

2. Material and Methods

This paper is largely based on studies that have been conducted in the forest/savannah transitional agro-ecological zone of Ghana between 2002 and 2006 under the framework of Convergence of Science project [10] and between 2007 and 2010 under the IDRC funded Climate Change Adaption programme in Africa [11]. Results from the 2002–2006 studies have already been published [8, 9, 12], while results from 2007–2010 studies have not been published.

2.1. The Study Area and Population. The studies were conducted in the Wenchi Municipal (7° 27 and 8° 30 N, 1° and 2° 36 W) in the forest/savanna transitional agro-ecological zone of Ghana. The relief of Wenchi is gently undulating to flat. The soils, which are mainly Lixisols, are fragile with shallow top soils underlain with compact concretions and impermeable iron pans [8]. Temperatures are relatively high with a monthly mean of about 30°C. Rainfall is bimodal and starts in April and ends in November with a dry spell in August. The rainy season is followed by a long dry season from November to March. The annual rainfall is about 1,300 mm with about 107 rainy days. Wenchi municipal, which has a total population of 97,058 (2000 census), is ethnically diverse with about 20% of the population being migrants from the three northern regions of Ghana and the neighbouring Burkina Faso.

2.2. Research Approach and Methods. In 2002, we carried out a study under the Convergence of Sciences project to explore farmers' soil fertility management strategies and their relevant social context [8]. In order to ground the research in the needs of the farming communities, a diagnostic study was carried out in the study area between July, 2002 and July, 2003 using Participatory Rural Appraisal tools such as drawing of a community territory map (to identify the differences in soil fertility patterns), a transect walk (to reveal the diversity of the landscape), and analysis of soil fertility management strategies and group discussions. Group discussions with 10–40 farmers were held in the village centre and/or on farmers' fields.

In addition, two sets of individual interviews with farmers were conducted to collect qualitative and quantitative data. In the first interview, which involved 40 farmers, the selection of farmers was done through stratified sampling. A list of farmers in the community was obtained from the village committee secretary and every tenth name from the list was selected for individual interviewing. The second interview which involved 38 farmers was conducted later to look at the farming characteristics of the various subcommunities in the village using a wealth ranking exercise. For this interview, 6–10 persons were selected from each wealth category within each subcommunity. The individual interviews were semistructured in nature and served both to get more quantitative data on farm size, household composition, and the farming system and to obtain a better qualitative understanding of the soil fertility management strategies and their underlying rationale.

The diagnostic study was followed by farmer participatory on-farm experimentations with three farmer research groups established soon after the diagnostic study to evaluate the agronomic efficacy of the soil fertility mana-gement practices being used by the farmers [9]. Six cropping sequences: cassava cropping; pigeonpea cropping; mucuna/maize/mucuna rotation; cowpea/maize/cowpea rotation; maize/maize/maize; *Imperata cylindrica* fallow were evaluated on both farmer-managed and researcher-managed plots for their effects on soil fertility and yield of subsequent maize test crop. To deepen our understanding of soil fertility management, we carried out further exploration of diversity among the farmers according to gender, ethnicity, and wealth [12]. Farmers were selected from three communities in Wenchi according to ethnicity and gender for interview using semistructured questionnaires. We conducted two sets of interviews. For the first interview, the native households were categorized into male-headed and female-headed households. Subsequently, a stratified sample was selected consisting of 20 males from male-headed households, 20 females from male-headed households, and 20 females from female-headed households. In the case of the migrants, every farmer in the community was interviewed because of the small size of their population. As migrant women do not have their own farming enterprises, only males were interviewed. In the second interview, the farmers were selected through a wealth ranking exercise. Fifteen farmers were selected from each of three wealth categories for interviewing. In addition, focus group discussions were held with chiefs, community leaders, family heads, and opinion leaders about land tenure systems in Wenchi.

In 2008, under the IDRC, funded climate change adaptation in Africa project, farmers evaluated three early maturing and three late maturing pigeonpea varieties obtained from the International Crop Research Institute of the Semi-Arid Tropics (ICRISAT) in India, in a community in Wenchi called Asuoano.

3. Results and Discussion

3.1. Farmers Views on Soil Fertility Decline and Their Causes. Farmers used several terms to express the fertility status of their soils although the terminology differed from one ethnic group to another. For instance, among the Akans and the Walas, when the soil loses its fertility, the farmers would say the soil is "tired" and therefore must be allowed to "rest" under bush fallow to regain its lost "energy." Farmers used various indicators such as colour, water holding capacity, and soil texture to assess soil fertility. A black soil was considered fertile while gravelly and sandy soils were considered less fertile. Other indicators used by farmers to express soil fertility included presence of earthworm casts which the farmers call "earth worm faeces," growth of crops, decline in crop yield and proliferation of certain plant species and weeds. For instance, the presence of weeds like *chromolaena odorata* and elephant grass (*Pennisetum purpureum*) indicated a fertile soil while the presence of weeds like spear grass (*Imperata cylindrica*) indicated a less fertile soil.

Farmers pointed out five major factors as being responsible for soil fertility decline in the area as follows: (1) increasing population as a result of migration of people into the area leading to smaller farms, which has resulted in the continuous cropping of the same piece of land over a long period; (2) annual bushfires which destroy vegetation and the population of earthworms that contribute to soil fertility improvement; (3) monocropping of maize without rotating it with any other crop; (4) the rapid increase in the monetary value of land; (5) felling of trees which exposes the land to the direct action of the sun.

3.2. Commonly Grown Crops in the Area. Table 1 shows the major food crops and grain legumes grown in the area. In terms of magnitude, maize was the most important food crop grown in Asuano. Other food crops included cassava, yams, cocoyam, and plantain. Legumes also played important role in the cropping systems in the area. Farmers listed about seven different types of legumes they cultivated (Table 1). In terms of proportion of land allocated to legumes, farmers ranked cowpea as the most important legume, followed by groundnut and pigeonpea. However, the type of legumes cultivated by farmers differed among the natives and the migrants. While the native farmers preferred pigeonpea, the migrant farmers preferred cowpea and groundnut. This was related to dynamics around land tenure. In Wenchi area, migrants do not own land but access land for farming through share cropping and land renting arrangements and hence preferred short duration legumes like cowpea and groundnut. Long duration legume such as pigeonpea was therefore not a preferred option for migrant farmers. Moreover, the market for pigeonpea is not well developed as it is a local food crop. It must be noted that the sum of the percentage number of people cultivating the various food crops added up to more than 100% because some farmers cultivated more than one crop since smallholder farmers in Ghana in general and Wenchi in particular practise mixed cropping. Yam was planted on mounds to increase the volume of soil for the development of tubers. Maize, cocoyam, and cassava were usually planted on the lower portions of the yam mounds while pigeonpea was planted in the rows between the yam mounds in a sequential order for efficient utilization of nutrients. Yam was usually planted first followed by maize, cocoyam, cassava, and lastly pigeonpea. The legumes mainly cowpea, groundnuts, and pigeonpea were grown to serve several purposes. These included (1) household food security; (2) household income; (3) soil fertility improvement through build up of soil organic matter and release of nutrients, particularly N for subsequent crops. Where crop rotation involving these legumes and maize was practised, crop succession was planned such that maize was planted after harvesting the legume to take advantage of the nitrogen fixed by the legume. In this way, it helped to either reduce or avoid the use of nitrogen fertilizers which may increase the cost of production.

Table 1: The most important food crops and proportion of land allocated to leguminous crops by native and migrant farmers in Asuoano in 2002.

	Natives	Migrants
	%	
Food crop		
Maize	100	100
Cassava	100	88
Yam	100	94
Cocoyam	91	19
Plantain	32	0
Leguminous crop		
Pigeonpea	50	0
Groundnut	20	35
Cowpea	10	50
White kidney bean	10	0
Bambara groundnut	5	15
Mucuna	2	0
Others	3	0

Source: [8].

3.3. Role of Pigeonpea in the Cropping Systems in Wenchi. Among the legumes cultivated by the natives, pigeonpea was grown on a larger scale in comparison with other legumes because of its ability to regenerate soil fertility, its low production cost, its tolerance to pests and diseases, its cash income, and its food value. It was generally grown on less fertile land and land with problematic weeds such as spear grass (*Imperata cylindrica*).

After cropping a piece of land to crops like maize, cassava, and yam for about three to four years, farmers intercropped their food crops with pigeonpea during the last cropping year of the cycle. After harvesting the component crops, farmers allowed the land to remain under pigeonpea for 18–24 months after which the pigeonpea plants were cut down, burnt and the land cropped to maize or yams. The pigeonpea canopy was perceived to protect the soil from the direct action of the sun and therefore prevents the soil from becoming hardened. According to the farmers, pigeonpea

Table 2: Effect of crop sequence and N rate on (a) maize grain yield (kg ha^{-1}) and (b) weed biomass (kg ha^{-1}) associated with the maize crop at 8 weeks after planting on researcher-managed plots.

(a)

Crop sequence	N rate (kg ha^{-1})		Mean
	O	60	
Speargrass fallow	1050	2848	1949
Cassava	3002	2738	2870
Pigeonpea	2422	2972	2697
Cowpea-maize-cowpea	1670	2328	1999
Mucuna-maize-mucuna	2970	4195	3582
Maize-maize-maize	1380	2128	1754
Mean	2082	2868	

SED: crop sequence (CS) = 318.4; N rate (NR) = 115.3; CS × NR = 375.8.
P < F: CS = 0.001; NR = 0.001; CS × NR = 0.01.
Source [9].

(b)

Crop sequence	N rate (kg ha^{-1})		Mean
	O	60	
Speargrass fallow	585	790	686
Cassava	270	300	285
Pigeonpea	390	500	445
Cowpea-maize-cowpea	325	395	360
Mucuna-maize-mucuna	300	345	323
Maize-maize-maize	240	430	335
Mean	351	460	

SED: crop sequence (CS) = 65.9; N rate (NR) = 52.4; CS × NR = 112.1.
P < F: CS = 0.001; NR = 0.05; CS × NR = NS.
Source [9].

forms a canopy after one year and shades out obnoxious weeds by suppressing their growth. The farmers also explained that the leaf litter covers the soil, reduces soil erosion, improves infiltration, prevents heating of the soil, and enhances earthworm activity. Crops grown on the land after pigeonpea, and especially maize, were perceived by the farmers to look greener, grow faster, and yield more. Rotations involving pigeonpea was often regarded by farmers as a long-term soil fertility management strategy. Despites its importance, migrant farmers preferred to use mainly short-term strategies such as mounding and the planting of short duration leguminous crops such as cowpea and groundnut for maintaining soil fertility because of the dynamics surrounding land tenure.

3.4. Agronomic Potential of Pigeonpea. Studies in Wenchi and Kade in the forest/savanna transitional and semideciduous forest zones of Ghana, respectively, indicate the potential of pigeonpea in improving soil fertility and farm profitability (Tables 2 and 3). In the transitional zone, rotating pigeonpea with maize resulted in about 75% increase in maize yield over the continuous maize control. In the semi-deciduous forest zone of Ghana, including pigeonpea in the cropping system also resulted in about 100–200% increase in maize grain yield over continuous maize. In the transitional zone, pigeonpea yielded about 25.5 tons of shoot biomass within 16 months of crop growth. High amounts of leaf litters of up to about 2 t ha^{-1} in one season could be considered a potential source of nutrients for subsequent crop as confirmed by a 75% increase in maize yield in the transitional zone and up to 200% increase in maize yield in the semi-deciduous forest zone of Ghana.

Table 3: Effect of crop sequence on maize grain yield at 12% moisture content.

Crop sequence	Maize grain yield (t ha^{-1})	
	2007/2008	2008/2009
Pigeonpea	7.0	2.3
Ex-Subi	4.6	1.9
Boakentemma	3.5	1.4
Cowpea-Maize-Cowpea	3.0	1.5
Groundnut-Maize-Groundnut	2.7	1.6
Maize-Maize-Maize	2.3	1.3
LSD at 5%	1.4	0.58

Source [13].

Higher maize yield under legume-maize rotation is expected since legumes are known to fix nitrogen, thereby improving the soil nitrogen economy and enhancing the growth of subsequent crop [6, 14, 15]. The higher maize grain yield associated with pigeonpea could largely be attributed to the large amounts of recycled N incorporated into the soil just before planting the maize test crops. The vigorous root system of pigeonpea has the capacity to explore large soil volume and recycles nutrients from deeper soil profiles. The faster decomposition of the biomass of pigeonpea and N release compared with that of maize stover was better synchronized with maize demand than the slower release of N by the poorer quality materials like maize stover. The N release patterns of organic residues of differential quality have been discussed extensively by [16, 17]. According to [16, 17], poor quality organic residues, with high C : N ratio, provide abundant supply of C for microbial growth leading to immobilization of soil N in the microbial biomass. Residues of high quality organic inputs on the other hand decompose quickly and may release about 70% of the N within a season under tropical conditions [18].

Besides the beneficial effect of pigeonpea on subsequent maize crops as a result of the high N recycling properties of the pigeonpea, the role of other nutrients cannot be ruled out. In some studies, increased soil P availability under pigeonpea was attributed to the efficient solubilisation and uptake of P from bound sources (e.g., Fe-P) by root exudates [19]. Benefit/cost analysis of different cropping sequences involving three grain legumes and cassava over four cropping seasons indicated that pigeonpea/maize rotations had 108% return on investment compared with 31% by continuous maize when fertilizer was not applied to the maize crop (Table 4).

3.5. Challenges in the Cultivation of Pigeonpea. Barrier to widespread adoption of pigeonpea include land tenure, market, and availability of early maturing varieties. In the

Table 4: Estimated costs of production, gross revenue, and returns on investment of (a) various crop sequences (b) maize grown after the sequences with N application to the maize and (c) maize grown after the sequences without N application to the maize.

Crop sequence	Economic yield (kg ha^{-1})	Total revenue (US$) ha^{-1}	Cost of production (US$) ha^{-1}			Total cost	Net revenue	Return on investment
			Land	Input	Labour			
(a) Crops in the sequence								
[1]Cassava	31,000	2545.1	41.7	41.7	635.0	718.4	1826.7	254
[2]Pigeonpea	1,870	623.3	41.7	8.3	221.5	271.5	351.8	130
[3]Mucuna-maize-mucuna	2,016	365.1	41.7	41.7	247.4	330.8	34.3	10
[4]Cowpea-maize-cowpea	2,536* (1,230)	1079.0	41.7	106,1	475.1	622.9	456.1	73
[5]Maize-maize-maize	3,287	595.2	41.7	36.1	386.1	463.8	456.1	28
[6]Speargrass fallow	0	0	41.7	0	0	41.7	−41.7	−100
(b) Maize after crop sequence with N application								
CS 1	2,738	495.9	13.9	104.2	190.2	308.3	187.6	61
CS 2	2,974	538.5	13.9	104.2	196.5	314.6	223.9	71
CS 3	4,194	759.4	13.9	104.2	245.9	364.0	395.4	108
CS 4	2,331	422.1	13.9	104.2	177.0	295.1	127.0	43
CS 5	2,126	385.0	13.9	104.2	175.4	293.5	91.4	31
CS 6	2,848	515.7	13.9	104.2	224.4	342.5	173.3	51
(c) Maize after crop sequence without N application								
CS 1	3,000	543.2	13.9	13.9	175.6	203.4	339.8	167
CS 2	2,423	438.8	13.9	13.9	165.5	193.3	245.5	127
CS 3	2,961	537.7	13.9	13.9	209.7	237.5	300.2	126
CS 4	1,772	302.8	13.9	13.9	155.2	183.0	119.8	66
CS 5	1,380	249.9	13.9	13.9	153.0	180.7	69.1	38
CS 6	1,048	189.8	13.9	13.9	173.7	200.9	−11.1	−6

[1] US$82.1 t^{-1}.
[2]US$333.3 t^{-1}.
[3]US$181.1 t^{-1} for maize.
[4]US$337.5 t^{-1} for cowpea and US$181.1 t^{-1} for maize.
[5]US$181.1 t^{-1} for maize.
[6]US$0.
*Yield of maize (figure in brackets).
CS 1: cassava; CS 2: Pigeonpea; CS 3: Mucuna-maize-mucuna; CS 4: Cowpea-maize-cowpea; CS 5: Maize-maize-maize; CS 6: Speargrass fallow.
Source: [9].

transitional zone of Ghana where the crop is widely cultivated by farmers, migrant farmers prefer cowpea cultivation to pigeonpea cultivation although they acknowledge the superiority of the latter over the former in terms of soil fertility management and the yield of subsequent maize crop (Table 5). According to the migrants, the market for pigeonpea is not always readily available and return on investment is too slow. In addition, they were afraid that when they invest in the soil, they would not be allowed to reap the full benefit. Among the native farmers, female and male farmers differed in the cultivation of legumes such as pigeonpea and cowpea. Women farmers preferred pigeonpea over cowpea due to its role in food security as well as its low labour requirement [12]. Male farmers preferred cowpea over pigeonpea because the market for cowpea is developed than that of pigeonpea.

Access of farmers to early maturing varieties is also another barrier to widespread adoption of the pigeonpea. Although the late maturing indeterminate pigeonpea varieties have the higher potential to improve soil fertility than the early maturing varieties due to greater biomass production, farmers prefer the early maturing determinate varieties. Criteria farmers used for selecting varieties for planting included maturity, plant height (for ease of harvesting), tolerant to insects, plant architecture (prefer varieties with smaller plant canopy for ease of intercropping with yams), and seed size (bigger seed size is preferred). Amount of biomass and/litter falls and ability to improve soil fertility were not mentioned as criteria although farmers claimed that soil fertility was one of their major production challenges and that pigeonpea was one of the crops they used to improve the fertility of their soils. Although late maturing indeterminate pigeonpea varieties with woody stems are a potential source of fuel wood, farmers did not also include this in their selection criteria. This may be due to the fact that accessibility to fuel wood was not a major constraint in Wenchi.

Table 5: Preferential ranking of different soil fertility management practices by native and migrant farmers in Wenchi.

Management practice	Ranking order*							
	Natives				Migrants			
	Asuoanoa $N = 10$	Beposob $N = 5$	Drobosoc $N = 7$	Average	Asuoanod $N = 6$	Beposod $N = 6$	Drobosoe $N = 5$	Average
(a) Ranking by natives and migrants								
Cassava	1	1	1	1	2	2	1	1.7
Pigeonpea	2	5	2	3	4	4	4	4
Mucuna/Maize/Mucuna	7	6	4	5.7	5	6	6	5.6
Groundnut/Maize/Groundnut	4	3	3	3.3	3	3	3	3
Cowpea/Maize/Cowpea	3	2	5	3.3	1	1	2	1.3
Maize/Maize/Maize	8	7	6	7	7	7	7	7
Cowpea/Cowpea/Cowpea	5	4	7	5.3	6	5	5	5.3
Bush fallow	6	8	8	7.3	8	8	8	8
(b) Ranking by female and male Bonos	Females $N = 13$	Males $N = 10$						
Cassava	1	1						
Pigeonpea	2	3						
Mucuna/Maize/Mucuna	5	7						
Groundnut/Maize/Groundnut	3	4						
Cowpea/Maize/Cowpea	4	2						
Maize/Maize/Maize	8	8						
Cowpea/Cowpea/Cowpea	7	5						
Bush fallow	6	6						

[a]Consisted of 6 males and 4 females; [b]Consisted of 4 males and 1 female; [c]Consisted of 6 females and 1male; [d]Dagarbas; [e]Walas.
*Each treatment was compared directly against the other until they were ranked from the highest to the lowest with 1 being the highest ranking and 8 being the lowest.
Source: [9].

During focus group discussions in the field, the farmers noted that the late maturing pigeonpea varieties had produced a lot of biomass and had higher litterfalls compared with the early maturing varieties. The farmers observed and commented on the greater litterfall from the late maturing varieties. They further stated that the greater production of biomass and the higher litterfall of the late maturing varieties would lead to better soil fertility improvement compared with the early maturing pigeonpea varieties. Despite their recognition of the soil fertility benefits of the late maturing varieties in improving soil fertility, the farmers did not include this as an important criterion for selecting which pigeonpea varieties to grow (Table 6). Mapfumo et al. [20] made a similar observation in Zimbabwe and suggested that pigeonpea is less likely to be adopted merely on the basis of its capacity to improve soil fertility. Farmers were thus interested in pigeonpea as a food security crop; soil fertility benefits were secondly. Farmers therefore opted for short duration/early maturing varieties which they could easily crop twice in a year. Thus the varieties ICPL 88039 and ICPL 88034 were the most preferred varieties because of their early maturity and high yielding. Although the variety ICPL 87091 was also preferred for their larger grain size, this variety was very susceptible to pod borers.

3.6. Implication of Large Scale Cultivation for System Sustainability. In addition to food uses, pigeonpea has outstanding soil improvement and conservation properties. The growth habit facilitates soil protection, as the canopy continues to expand during the dry season after the component crops in the mixed cropping have been harvested. Living and senesces pigeonpea leaves may offer protective cover for the soil during dry season to prevent the soil from drying out and reduce soil erosion and enhance rain percolation during the rainy season. In farming systems with minimal application of external inputs, management of organic resources plays a major role in maintaining both nutrient availability and soil organic matter [21]. In a cereal-based farming system as that found in the forest/savanna transitional agro-ecological zone of Ghana, where external input use is minimal, most recycling of N and P occurs through pigeonpea litterfall and green leafy biomass of pigeonpea incorporated into the soil after pigeonpea harvest [9]. Pigeonpea litterfall and green leafy biomass of pigeonpea are important sources of easily mineralizable N due to their high nitrogen (1.9 and 2.2% for litterfall and green leafy biomass, resp.) leading to high decomposition rates. Thus, rotation involving pigeonpea within smallholder agriculture has the potential of maintaining a reasonable supply of N and P to cereal crops,

Table 6: (a) Farmers' preference ranking and (b) farmers' criteria for selection of pigeonpea varieties for planting.

(a) Variety	Growth and yield characteristics					Ranking and reasons for the rank	
	Flowering pattern	Maturity period (days)	100 grains weight (g)	Yield (tons ha^{-1})	Seed colour	Ranking	Reasons for the rank
ICPL 88039	Determinate	125	10.1	1.6	Brownish red	1	Early maturing, short plant height, could be planted twice in a year
ICPL 88034	Determinate	125	10.2	1.8	Brownish red	2	Early maturing, short plant height, could be planted twice in a year
ICPL 87091	Determinate	125	12.1	1.1	white	3	Early maturing, short plant height, could be planted twice in a year
Maruti	Indeterminate	190	10.0	NA	Brownish red	4	Medium maturity, high litter fall
Lakshmi	Indeterminate	196	10.2	NA	Brownish red	4	High litter fall
ICPL 96053	Indeterminate	195	10.1	NA	Brownish red	4	High litter fall

(b) Criteria	Ranking
Maturity	1
Number of plantings in a year	2
Easy of harvesting (plant height)	3
Easy of intercropping with yams	4
Seed size	5

NA: not available.
Source: Adjei-Nsiah (unpublished results).

particularly maize considering the minimal use of external inputs in a maize-based farming system.

4. Conclusion

The paper shows the potential of pigeonpea in improving soil fertility in the smallholder farming systems particularly in a predominantly maize-based farming system as that found in Wenchi, Ghana. We have shown that the potential of pigeonpea in sustaining the productivity of predominantly maize-based farming system is due to its nutrient recycling properties as well as to its role in food security and flexibility in external input use and labour requirement. Soil fertility was apparently not a major criterion during varietal ranking by farmers suggesting that pigeonpea is less likely to be adopted merely on the basis of its capacity to improve soil fertility. Even when there was no strong market demand for pigeonpea, female farmers still integrated pigeonpea in their rotational system. Pigeonpea is largely a women's crop and therefore the attitude by men as major household decision makers has probably undermined its adoption in a predominantly maize based cropping system in Wenchi. Empowering women to make decision with respect to allocation of production resources may enhance a greater adoption of legumes. As more farmers resort to putting their land under pigeonpea instead of bush fallowing, pigeonpea cultivation could serve as an entry point for farming system sustainability. There is, however, the need to (i) introduce short duration pigeonpea varieties that will fit well in the predominantly mixed cropping systems of smallholder agriculture; (ii) evaluate the nutrient recycling capacities of different pigeonpea genotypes; (iii) develop crop rotation/sequencing and soil management options that can improve and/or sustain the productivity of pigeonpea through integrated soil fertility management (ISFM); (iv) design a range of social arrangements that will encourage investment in soil fertility through integration of pigeonpea in the farming system in a heterogeneous farming community like Wenchi, (v) develop use for pigeonpea in new cooking options and/or value added products.

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