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Seed shape in model legumes: Approximation by a cardioid reveals differences in ethylene insensitive mutants of *Lotus japonicus* and *Medicago truncatula*

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ABSTRACT

Seed shape in the model legumes *Lotus japonicus* and *Medicago truncatula* is described. Based in previous work with Arabidopsis, the outline of the longitudinal sections of seeds is compared with a cardioid curve. *L. japonicus* seeds adjust well to an unmodified cardioid, whereas accurate adjustment in *M. truncatula* is obtained by the simple transformation of scaling the vertical axis by a factor equal to the Golden Ratio. Adjustments of seed shape measurements with simple geometrical forms are essential tools for the statistical analysis of variations in seed shape under different conditions or in mutants. The efficiency of the adjustment to a cardioid in the model plants suggests that seed morphology may be related to genome complexity. Seeds of ethylene insensitive mutants present differences in size and shape as well as altered responses to imbibition. The biological implication and meaning of these relationships are discussed.

Introduction

The description of plant form and shape is useful for understanding evolutionary relationships and quantifying differences between species, as well as for defining the processes of plant development over space and time. The scientific discipline of *plant morphology* (Kaplan, 2001), however, should be mathematically, and not solely descriptively, based. Seed spacing in a sunflower head, or rings swirls on a pineapple fruit, whose distribution follows the Fibonacci series, are good examples, while others await discovery. Computational modeling of plant development has been applied to legume lateral root development (Han et al., 2011) as well as nodule pattern (Han et al., 2010). Such a mathematical and computational basis permits the *in silico* testing of thousands of experimental conditions to achieve outcomes which then need to be verified in 'real world' experiments.

Recently, we described a mathematical–geometrical model to quantify the shape of *Arabidopsis* seeds. The model consisted of the comparison of the outline of the longitudinal equatorial section of the seed with a transformed cardioid (Cervantes et al., 2010). The cardioid is the trajectory described by a point of a circle that rolls around another fixed circle with the same radius, and transforming it by multiplying the x-axis by the scaling factor (1.61803399), the so-called 'Golden Ratio.' The figure obtained resembled the image of an *Arabidopsis* dry seed. The similarity was evaluated by a factor, the G index, allowing the precise morphological description of genotypes and opening new ways to examine the association between genetic changes and alterations in the morphology, *i.e.*, a classical genome–phenome analysis.

Modeling the *Arabidopsis* seed shape by a transformed cardioid allowed comparison between genotypes showing that ethylene insensitive *etr1-1* mutants presented alterations from the wild type and suggesting that ETR1 (one of the histidine protein kinase ethylene receptors) function goes beyond its role in ethylene signal transduction (Cervantes et al., 2010). The functional connection between ethylene perception and seed shape is unclear, but may involve diverse aspects of seed development as well as drying rates.

Legume seeds are an excellent material with which to expand this type of analysis in search of accurate morphological descriptions. Legumes are a large family containing members of great agronomic interest (*e.g.*, soybean, pea, common bean, medics, clovers as well as trees such as Acacias, *Robinia* and *Pongamia*; Biswas et al., 2011). In the context of seed morphology, legume species are characterized by remarkable variation, ranging from small seeds such as *Lotus japonicus* to large-sized seeds of *Phaseolus* or *Vicia* and gigantic ones (7–8 cm long) in the Black Bean Tree (*Castanospermum australe*) of Australia.

Interestingly, *L. japonicus* and *Medicago truncatula*, two model species intensively used in genomic analysis (Jiang and Gresshoff, 1997; Sato et al., 2008; Cannon et al., 2006; Young et al., 2005),

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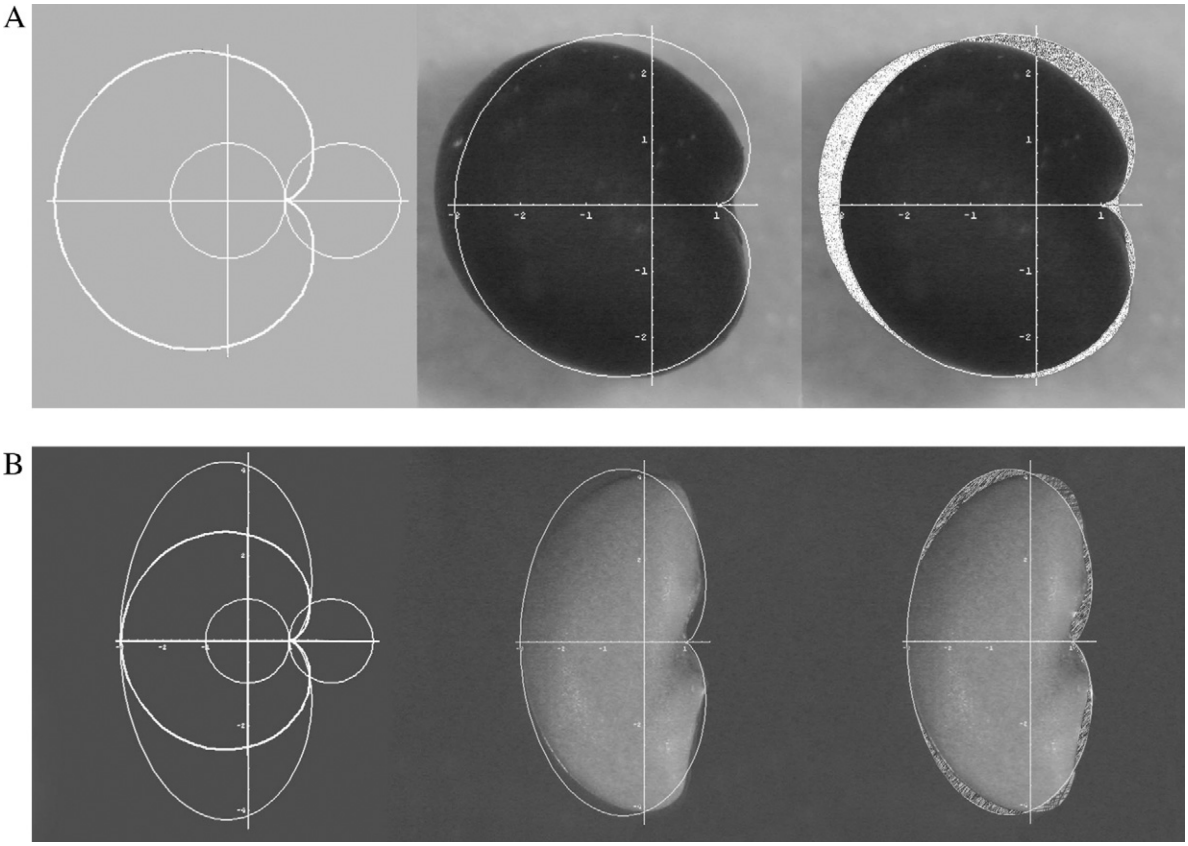

Fig. 1. (A) Adjustment of the bi-dimensional image of dry seeds of *Lotus japonicus* var. Gifu to a cardioid. The cardioid curve is shown on the left. In the center, photographic images of *Lotus* seeds were superimposed to the cardioid. For the quantification of the adjustment areas in two regions were compared: the common region to the cardioid and the seed image and the regions not shared between both figures (figure on the right, striped). (B) Adjustment of the bi-dimensional image of dry seeds of *Medicago truncatula* var. Jemalong (A-17) to a cardioid scaled in the vertical axis by a factor of φ. The cardioid curve and modifications are shown on the left. In the center, photographic images of *Medicago truncatula* seeds were superimposed to the cardioid. Quantification of the adjustment was as for Lotus (see (A)).

are amenable to morphological analysis based on a simple model resembling the one described for *Arabidopsis*, *i.e.*, the comparison with a cardioid. Whereas the seed dimensions of *L. japonicus* match well to a cardioid, in *M. truncatula*, accurate adjustment is obtained by the simple transformation of scaling the vertical axis by a factor equal to the Golden Ratio. As in the case of *Arabidopsis*, the application of these models allows the description of alterations in the ethylene response mutants.

Materials and methods

Plant material

Seeds of *Lotus japonicus* of the ecotypes Gifu and Miyakojima (MG-20) were used. MG-20 is a Japanese ecotype from a more tropical habitat than Gifu. The former comes from the Miyakojima Islands south of the Japanese island chain, and the latter from the Gifu prefecture close to Tokyo. They differ in their ABA sensitivity to germination and other photoperiod-related traits (Biswas et al., 2009). *LjETR1-1* is a transgenic line obtained from the stable transformation of Gifu with the dominant *Arabidopsis* ethylene receptor gene *ETR1-1* (Lohar et al., 2009). Mutant *enigma-1* is one of three independent mutants of the *Lotus EIN2a* gene in the MG-20 background produced by EMS mutagenesis and M2 selection for ethylene insensitivity in seedling root growth (Chan et al., in press). *Ljhar1-1* is a mutant of the Gifu variety in *HAR1*, a gene involved in the systemic regulation of nodulation and structurally related to the *Arabidopsis thaliana CLAVATA1* (*CLV1*) gene (Krusell et al., 2002; Nishimura et al., 2002; Wopereis et al., 2000). Seeds of *Medicago truncatula* wild type A17 as well as the ethylene insensitive and increased nodulation mutant *sickle* (Penmetsa et al., 2008) were used. Of interest is the fact that both *sickle* and *enigma-1* are mutated in *EIN2*, an integral component of the *Arabidopsis* ethylene sensing pathway.

Photography and image analysis

The seeds were observed with a Nikon ‘SMZ-2T’ stereomicroscope. Photographs of longitudinal views were taken with a digital camera Nikon ‘Coolpix 950’ and analyzed with the software image Photoshop CS4 (Adobe), specialized for image processing. In this process, graph paper was used to convert pixel into micrometer.

Size and shape analysis

The area of the seed images was obtained with Photoshop CS4 and used as the measure of seed size. Circularity index (Schwarz, 1980), given by

$$I = 4\pi \frac{area}{(perimeter)^2}$$

is a measure of the similarity of a plane figure to a circle. It ranges from 0 to 1, yielding a value of 1 for circles, and it is a useful magnitude as a first approximation to seed shape. Eight independent experiments with 10 seeds for each variety or genotype were used

Table 1
(A) Mean values for area (mm^2) of dry and imbibed seeds in *Lotus japonicus* genotypes are given. (B) Mean values for circularity index *I* of dry and imbibed seeds in *L. japonicus* genotypes. (C) Mean values for *J*-index of dry and imbibed seeds in *L. japonicus* genotypes.

	Wild type		Mutants		
	MG-20	Gifu	*enigma-1*	*Ljetr-1-1*	*Ljhar1-1*
(A)					
Dry seeds	1.6	1.5	1.5	1.3	1.3
Imbibed seeds	3.8	3.2	3.1	2.6	2.8
(B)					
Dry seeds	0.82	0.82	0.82	0.82	0.82
Imbibed seeds	0.86	0.85	0.85	0.85	0.86
(C)					
Dry seeds	92.7	92.6	90.9	92.6	91.5
Imbibed seeds	93.5	90.9	89.8	88.5	92.4

in *L. japonicus*. For *M. truncatula*, three experiments with 10 seeds for each genotype were performed.

Quantification of the image adjustments to a cardioid

The cardioid is the trajectory described by a point of a circle that rolls around another fixed circle with the same radius (see Fig. 1A (left)). This common radius is the only parameter needed to define a particular cardioid. In Cervantes et al. (2010), the image of *Arabidopsis* seeds was shown to adjust well to a cardioid modified by elongating its horizontal axis by a factor equal to the Golden Ratio φ. The pictures of seeds were combined with cardioid (Fig. 1A; Lotus) or modified cardioid (Fig. 1B; Medicago) images.

Quantification of the adjustment was done in each seed by the comparison of the areas in two regions: the common region in the cardioid and the seed image, and the regions not shared between both areas (see Fig. 1). The index of adjustment (*J*) is defined by:

$$J = \frac{area(C)}{area(C) + area(D)} \times 100$$

where *C* represents the common region and *D* the regions not shared. Note that *J* is a measure of seed shape, not of its area. It ranges between 0 and 100, decreasing when the size of the non-shared region grows, and equals 100 when cardioid and seed image areas coincide, *i.e.*, when area (*D*) is zero.

The adjustment was done with an unmodified cardioid for *L. japonicus*. In Medicago seeds, the adjustment was improved by elongating the *y*-axis by a factor of φ (Fig. 1B). Shape comparison among genotypes was done by the statistical contrasting of their *J* index values in dry seeds as well as after 24 h of imbibition.

Statistical analysis

ANOVA was used for the comparison between different groups. *Post hoc* analysis was carried out using the Scheffé test for samples of heterogeneous size. Tukey's test was used for samples of similar sizes. Statistical analysis was performed using SPSS.

Results

Tables 1–4 contain the results from experiments with *L. japonicus*. The mean values for seed image area (Table 1A), circularity index (Table 1B), and *J* index (Table 1C) in the varieties and genotypes used are shown. Tables 2A, B and 3A, B present the contrast tests for seed image area and *J* index, while Table 4 contains a schematic representation of results. Table 5 contains values for area, circularity index and index *J* of dry and imbibed seeds in *M. truncatula* genotypes. The results are presented in Fig. 3.

Table 2
Contrast tests after ANOVA in *Lotus japonicus* genotypes. (A) Tuckey test showing differences between groups for the area of dry seeds and (B) Scheffé test showing differences between groups for the area of imbibed seeds.

Genotype	*N*	Subset $\alpha = 0.05$		
		1	2	3
(A)				
Ljhar1-1	94	1.28		
LjETR1-1	92	1.31		
Gifu	94		1.46	
enigma-1	95		1.47	
MG20	89			1.56
Significance		0.524	0.994	1.00
(B)				
Ljhar1-1	33	2.63		
LjETR1-1	31	2.84	2.84	
Gifu	26		3.15	
enigma-1	43		3.16	
MG20	32			3.81
Significance		0.574	0.135	1.00

Table 3
(A) Tuckey test showing differences between groups after ANOVA for *J*-index of dry seeds in *L. japonicus* genotypes. (B) Scheffé test showing differences between groups after ANOVA for *J*-index of imbibed seeds in *L. japonicus* genotypes.

Genotype	*N*	Subset $\alpha = 0.05$	
		1	2
(A)			
enigma-1	31	90.90	
Ljhar1-1	30	91.49	91.49
LjEtr1-1	30		92.61
Gifu	30		92.64
MG-20	30		92.74

Genotype	*N*	Subset $\alpha = 0.05$			
		1	2	3	4
(B)					
LjEtr1-1	33	88.48			
enigma-1	26	89.78	89.78		
Gifu	43		90.94	90.94	
Ljhar1-1	31			92.37	92.37
MG20	32				93.53
Significance		0.394	0.510	0.299	0.517

Adjustment of wild type L. japonicus seeds to the cardioid shape and comparison of size and shape between varieties

The images of Lotus seeds adjust to an unmodified cardioid both in dry seeds (Fig. 1A) and seeds imbibed for 24 h. There was no difference between the *J* index in dry seeds of Gifu (92.6%) and MG-20 (92.7%). In contrast, after imbibition, the *J* index was smaller in Gifu (90.9%) than in MG-20 (93.5%; Tables 1C and 3A, B; Fig. 3), perhaps reflecting differential ABA sensitivity in germination described by Biswas et al. (2009). Seeds of Gifu were smaller than MG-20, both in dry status and after imbibition (Tables 1A and 2A, B; Fig. 3).

Table 4
Differences in seed size and shape between varieties of Lotus (Gifu *vs* MG-20), or between mutants and their respective parental genotypes. MG-20 is the parental for *enigma-1*; Gifu, for *LjEtr1-1* and *Ljhar1-1*). (+) represents difference; (–), no difference.

	Size		Shape	
	Dry	Imbibed	Dry	Imbibed
Gifu *vs* MG-20	+	+	–	+
LjEtr1-1 vs Gifu	+	–	–	+
Ljhar1-1 vs Gifu	+	+	–	–
enigma-1 vs MG-20	+	+	+	+

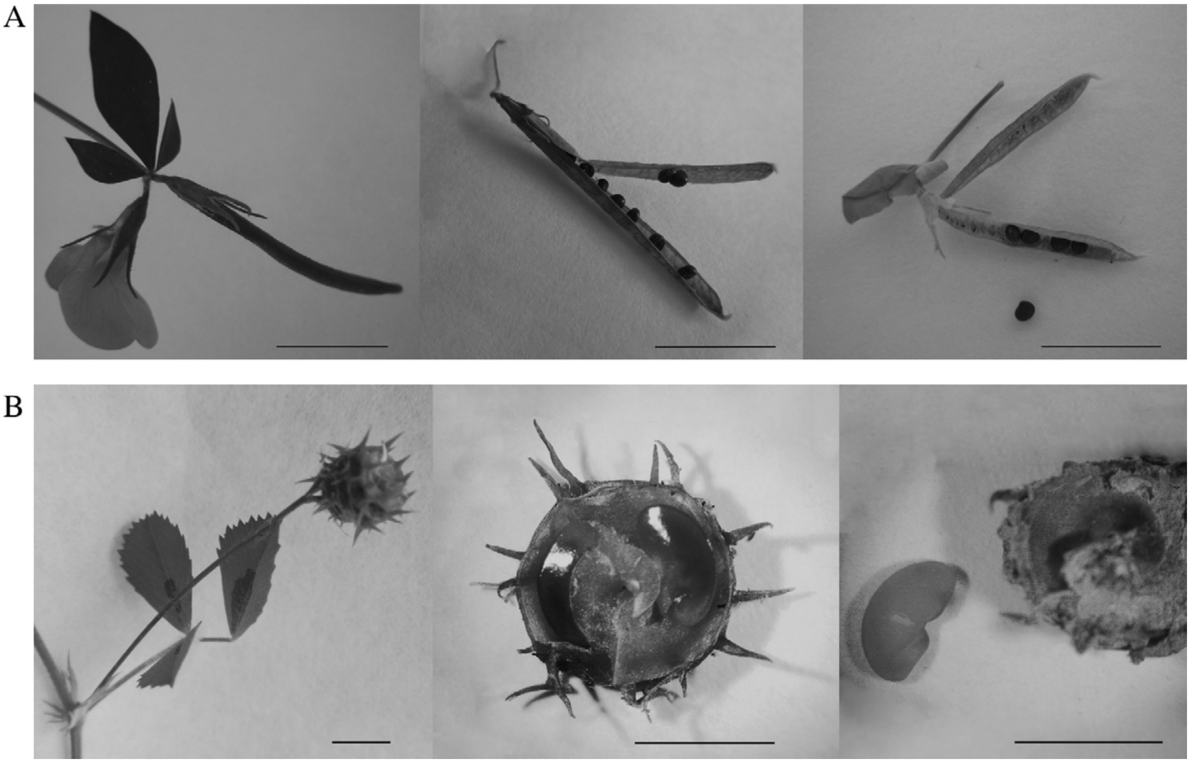

Fig. 2. Close up photo of pods and seeds: (A) *Lotus japonicus* (scale bar equals 1 cm). (B) *Medicago truncatula* (scale bar equals 5 mm).

Variation in size and form in L. japonicus mutant genotypes

The sizes and forms of seeds in genotypes Gifu, *LjETR1-1*, MG-20, *enigma-1* and *Ljhar1-1* were compared. MG-20 is the parent for *enigma-1*; Gifu, for *LjEtr1-1* and *Ljhar1-1*.

Dry seeds

Table 2A shows the results of the ANOVA comparing the areas of dry seeds. Gifu seeds were larger than those of *Ljhar1-1*, reflecting differences in plant vigor (see Wopereis et al., 2000). Mutant seeds in the ethylene pathway in both varieties were smaller than their corresponding wild type sources; that is, *LjETR1-1* seeds were smaller than Gifu, and *enigma-1* smaller than MG-20. No differences were detected in the values of the circularity index between wild type varieties or between each of the varieties and their respective mutants (ANOVA not shown). The *J* index was smaller in *enigma-1* than in MG-20 (Table 3A; Fig. 3). No differences in the *J* index were found between *Ljhar1-1* and *LjETR1-1* with their parental variety (Gifu).

Imbibed seeds

Analysis of imbibed seeds of Lotus genotypes revealed differences in size between genotypes (Table 2B). Gifu seeds were larger than their mutant genotypes *Ljhar1-1* and *LjETR1-1*. Seeds of MG-20 were larger than *enigma-1*, which is consistent with difference in pod and plant size (Chan et al., in press); no differences were observed between genotypes in their circularity index. After imbibition, the *J* index values were smaller for mutant genotypes in the ethylene sensing pathway: *enigma-1* had smaller values than MG-20, and *LjETR1-1* was smaller than Gifu (Table 3B). There was no difference between Gifu and *Ljhar1-1*.

Table 5
Mean values for area, circularity index and index *J* of dry and imbibed seeds in *Medicago truncatula* genotypes.

	Area		Circularity index		*J*-Index	
	A 17	*Sickle*	*A 17*	*Sickle*	*A 17*	*Sickle*
Dry seeds	5.3	4.7	0.72	0.70	87.1	86.0
Imbibed seeds	10.1	9.1	0.70	0.69	86.8	86.4

Comparison between dry and imbibed seeds

Area and circularity indexes increased in all varieties and genotypes upon seed imbibition (Table 1A and B), as indicated by *t*-test ($P < 0.05$; not shown). The *J* index decreased with imbibition in genotypes *LjETR1-1*, Gifu and *enigma-1* ($P < 0.05$; not shown). No differences were detected in MG-20 and *Ljhar1-1* (not shown).

Adjustment of wild type Medicago seeds to a cardioid

For the adjustment of Medicago seeds, departing from a cardioid the vertical axis was scaled by a factor equal to the Golden Ratio $\varphi \approx 1.618$ (Fig. 2B). The mean values for the *J* index in *M. truncatula* A17 were 87.1% and 86.8% for dry and imbibed seeds, respectively (Table 5). No differences were found between treatments.

Variation in size and form in Medicago genotypes

Dry seeds

Differences between wild type A17 and the *sickle* mutant were found in size (area) and circularity index, but not in the *J* index (Table 5).

Imbibed seeds

After imbibition, no differences were observed between the *Medicago* genotypes either in area, circularity or *J* index (not shown).

Comparison between dry and imbibed seeds

Imbibition resulted in increased areas and reduced circularity index values in both genotypes ($P < 0.05$; not shown). There was no change in the *J* index with imbibition (not shown).

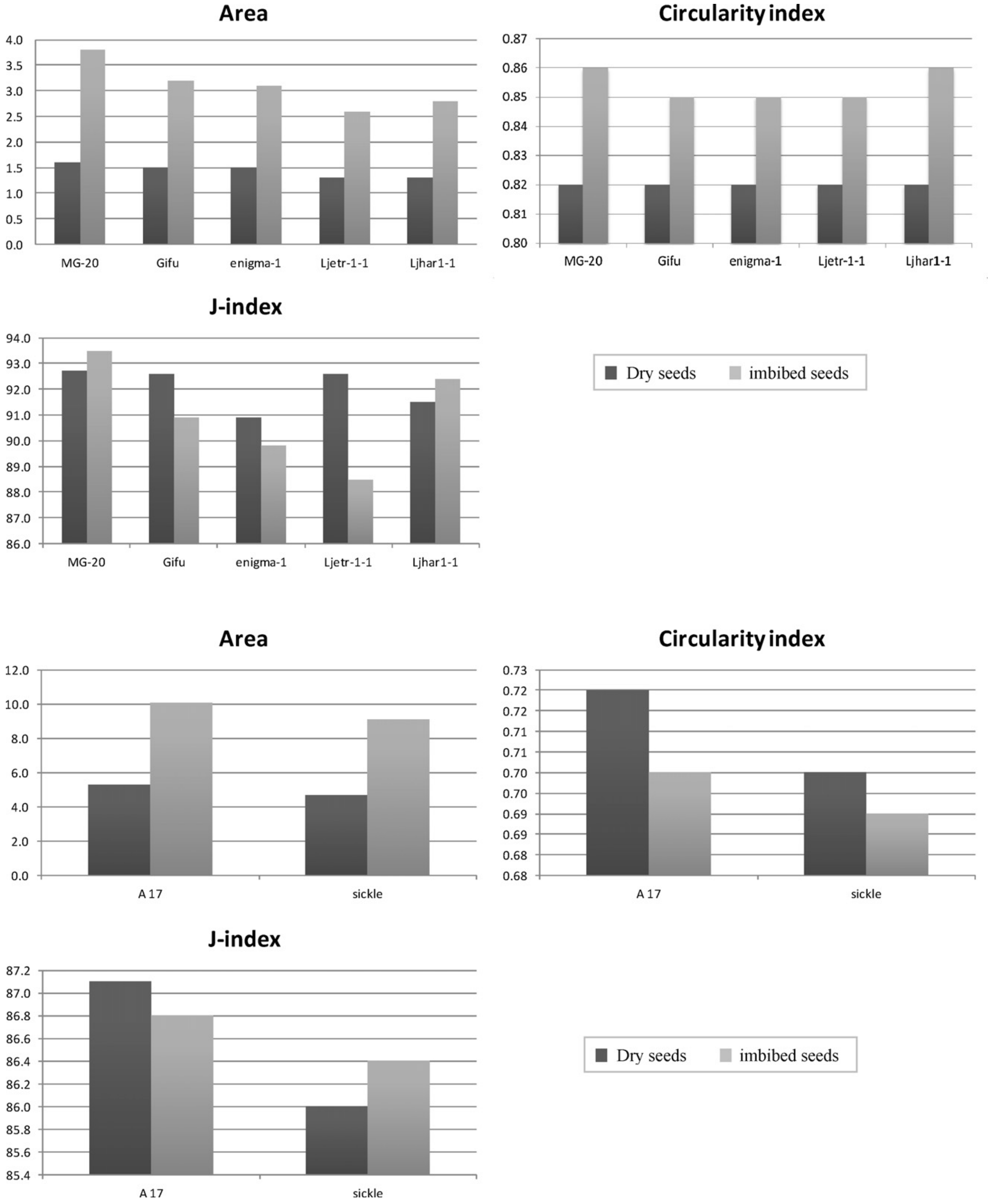

Fig. 3. Graphical representation of Area, Circularity index (*I*), and *J* index in genotypes of *Lotus japonicus* (A) and *Medicago truncatula* (B).

Discussion

The analysis of plant development requires an accurate and quantitative description of morphology. Space, volume, shape and surface interact as biophysical constraints with gene activity. Changes in size and shape may occur independently, as the results of developmental programs in a “regular” environment or in response to changes (stress) in environmental conditions (Cervantes and Tocino, 2009). The morphological analysis of seeds in model species may reveal quantitative measures and provide new parameters useful for the identification of genetic elements responsible for seed shape, a field of research that is independent from size, and that has remained virtually unexplored.

In a previous study, the image of a transformed cardioid was used as a model for the shape of *Arabidopsis* seeds. The transformation resulted in elongating the cardioid along the *x*-axis (*i.e.*, horizontally) by a factor of φ, the Golden Ratio (Cervantes et al., 2010). The model based in a cardioid curve better fits the shape of *Arabidopsis* seeds than a former model based on a prolate spheroid (Robert et al., 2008), and it is applied here to seeds of the model legumes *L. japonicus* and *M. truncatula*.

Lotus seeds are rounded and their shape is best described by the model of an unmodified cardioid, whereas for Medicago, the cardioid needed to be elongated by a factor of φ in the vertical direction to provide a fit. The seeds of *Arabidopsis* and Medicago present an elongated form according to their growth inside the pods. The final seed shape resembles a modified cardioid. In *Arabidopsis*, the seed presents the micropyle in one pole and the cardioid is elongated in the horizontal direction. In *M. truncatula*, called Australian Barrel Medic, the pod is compacted into a fused spiral, and the seed develops inside leaving the micropyle in a central position (Fig. 2B), so the resulting cardioid is elongated in a vertical direction. In both species, *M. truncatula* and *Arabidopsis thaliana*, similar physical restrictions during seed development result in an optimized adjustment modifying the cardioid by a factor of φ. Lotus seeds, as many other legumes (soybean, pea, chickpea and lentil) develop independently inside an elongated pod (Fig. 2A), but are more rounded than those of Medicago or *Arabidopsis*.

In three model species (*Arabidopsis*, Medicago, Lotus), seed shape can be well-adjusted to simple geometric models: unmodified or slightly modified cardioid curves. Departing from these curves, it may be possible to design new geometric models adapted to other species in their families. Model species share important characteristics (short life cycles, small and abundantly produced seeds, lack of specialized forms). They represent examples of organisms with “type r” strategies and, thus, simple, easily described seed shape may be an important feature of model plants (Begon et al., 2006; Cervantes, 2008).

The comparisons between genotypes demonstrated differences in seed shape (based on the cardioid), as well as in size. Differences in ABA sensitivity between varieties Gifu and MG-20 have been previously reported (Biswas et al., 2009). Here, we found that MG-20, an ecotype more tropical and less sensitive to ABA during seed germination than Gifu, has larger seeds, suggesting that in *L. japonicus*, an inverse relationship may exist between seed size, latitude of origin and ABA sensitivity. An inverse relationship between seed size and latitude has been reported in general for plants (Moles et al., 2007), as well as in the legume family for seeds of *Glycine* species (Murray et al., 2003).

Antagonistic roles have been found for ABA and ethylene in seeds. In *Arabidopsis*, ethylene-insensitive *ein2* mutants have enhanced sensitivity to ABA (Beaudoin et al., 2000; Ghassemian et al., 2000). Seeds of *enigma* mutants have enhanced sensitivity to ABA (Chan et al., in press) that is associated with reduced seed size. ABA is known to promote quiescence and suppress differentiation in the *Arabidopsis* primary root meristem (Zhang et al., 2010). Thus, in *L. japonicus*, reduced seed size may result from limited cell division as a consequence of increased ABA sensitivity. Also, reduced size results in an increased ‘surface/volume’ ratio that may be responsible for higher ABA penetration in the cells. Finally, ABA insensitivity may involve a delay in pod abscission and maturation resulting in larger seeds.

Arabidopsis ethylene-insensitive mutants (*etr1-1*) presented differences in seed shape when compared to the wild type. Mutant seeds resemble less a cardioid, having lower values of the *G* (equivalent to the *J* index) index than wild type (Cervantes et al., 2010). The genotypes mutated in the ethylene sensing pathway in *Lotus* and *Medicago* presented a more complex range of alterations in seed size and shape.

In *L. japonicus*, differences in seed size were found both in dry seeds and after imbibition. In addition to the differences between varieties (MG-20 has larger seeds than Gifu), in general, seeds of the wild type varieties were larger than their corresponding mutants, resulting in a broad range of diversity for seed size in *L. japonicus*. Reduced seed size in the mutants may be associated with altered meristematic activity, this being a direct effect of the mutation in *Ljhar1-1* (Nishimura et al., 2002; Krusell et al., 2002), or a consequence of altered hormonal sensitivity (ABA and ethylene in the ethylene-insensitive mutants).

Differences in shape were also found. Dry seeds of MG-20 fit better to a modified cardioid than seeds of mutant *enigma-1* (the *J* index was smaller in *enigma-1* than the parent). The effect was maintained in imbibed seeds. Similarly, in imbibed seeds, the *J* index was also smaller in *LjETR1-1* than in its wild type parent Gifu. Thus, in *enigma-1*, reduced seed size was associated with altered shape both in dry and imbibed seeds (Table 4 and Fig. 3). In *LjETR1-1* both effects were associated in imbibed seeds (dry seeds of *LjETR1-1* were smaller than Gifu, but no differences in shape were observed). Finally, seeds of *LjHar1-1*, although of reduced size, did not present differences in shape with the parent (Gifu). Thus, the results allow discrimination between changes in size and shape during seed germination, and show different effects of the mutants in both characters. The effect of *enigma-1* mutants goes beyond seed size and involves shape alterations.

Mutants in the *EIN2* gene presented alterations in size and shape both in dry and imbibed seeds in *Lotus*, but only in size and circularity index (not in the *J* index), in dry seeds of *Medicago*. Mutants in this gene also have different effects in nodulation. *Medicago* mutant (*sickle*) developed increased nodule numbers per plant while Lotus mutant (*enigma*) had reduced or equivalent values (dependent on the allele). These nodulation differences may be accounted for by the presence of a second copy of *Lotus EIN2* (*LjEIN2b*; Desbrosses and Stougaard, 2011) on *Lotus* chromosome 5, suggesting pathway diversification after gene duplication. Our results add new data to the contrasting nodulation phenotypes indicating divergences between both model species.

Our present results allow us to distinguish two processes during seed germination, *i.e.*, seed growth and the development of shape (Table 4). Mutations in Lotus *EIN2a* gene (in *enigma-1*) have effects on seed size and shape. The fact that other mutations (*LjHar1-1*) have effects only on seed size, but not on shape indicates that both processes may involve different regulatory mechanisms. For example, seed size may be the result of cell size, seed filling, and cell division activity, processes being under the control of distinct metabolic pathways, whereas seed shape could be submitted to systemic regulation, cell extension, physical constraint, and regulation of polarity. The combination of these processes represents an interesting system for the analysis of hormonal action in plant morphology.

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