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Spatial constraints also regulates final achene mass in the sunflower (*Helianthus annuus* L.) capitulum

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Abstract

In capitula of the cultivated sunflower (*Helianthus annuus* L.) achene size and mass commonly decrease from proximal to distal positions. Temporal limitation of resources of the distal achenes over the proximal ones has been the common explanation for this response. Nevertheless, because the capitulum architecture and expansion dynamics also interacts with achene growth and development, also space exert a coupled effect with resources on achene size along the inflorescence radius. In this work we removed young achenes from different capitulum positions [inner sector (IS) and outer sector (OS)] and applied an artificial restriction to the capitulum/achenes radial expansion. Removal of outer achenes significantly increased the final dry mass of the remnant ones between 17.1 to 27.6%. Removal of inner achenes also produced the same effect but in less magnitude, between 9.3 to 17.9% of the outer ones. The removal of outer achenes with the application of an artificial peripheral constraint did not significantly increase the dry mass of the remnant ones (2.7% of the inner and 7.1% of the control). Percentage of empty achenes significantly diminished in the middle sector (MS) in capitula with the outer achenes removed and in capitula with the outer achenes removed plus a peripheral constraint but in the range of 7.1% (MS achenes) and 2.7 % (IS achenes). Percentage of empty achenes of the MS did not change when the outer achenes were removed but was significantly lower when the OS was removed and the peripheral constraint was applied. This results suggest that a part of the reduced growth and development of IS and MS achenes is not only controlled by the competition for resources but also is restricted by space and pressure exerted by the neighboring ones.

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Introduction

Sunflower grain yield is mainly determined by the number of achenes produced in the inflorescence (capitulum) and their average mass.[1,2] Also, in the production of hybrid sunflower seed, achene size and homogeneity are important determinants of seed quality.[3] Achene size and/or achene set diminution into an inflorescence have been to occur in several species.[4] These were attributed either to competition for resources,[5-7] or to architectural constraints such us reduced vascular ways to supply photoassimilates to the sinks.[8-10]

The physiological mechanisms that regulate these processes are complex. Nevertheless experiments made in the sunflower, where the source/sink relationship was changed, show that achene size differences along the radial length of the capitulum, *i.e.* from the periphery towards its center (peripheral to distal positions), are due from insufficient assimilate supply from the sources.[2,11-15] Besides these well documented evidences, few works on achene development in Asteraceae inflorescences have been focused on to the relationship of maximum achene mass or size and the positional incidence following achene or inflorescence growth. For example it has been observed in *Tragopogon porrifolius* L. (*Asteraceae*) that the removal of external achenes during capitulum development resulted in heavier internal achenes, while it was not the case when internal achenes were removed.[16] On the other hand it is known that into a developing capitulum, space for floret or achene development changes on time and can play a relevant role in controlling the final floret number or achene size and eventually achene mass. For example, in young sunflower plants, the external application of cytokinins,[17-19] or surgical removal of the involucral bracts at early stages of capitulum development,[18] both ways to increase the size or the meristematic surface of the still undifferentiated receptacle into floret primordia, significantly increased the number of florets and, at maturity, the number of achenes per plant.

The selective removal of flowers in different sectors of the sunflower capitulum, external, middle and internal, generated a significant increase in the mass of achenes of the inner region of the capitulum as well as reduced the number of empty achenes, while it was not so and the effect was much smaller with the reverse treatment.[20] In this case, the experiment was able to show the positional effect in terms of the demand for photoassimilate in different capitulum positions.

So not only available resources but also physical limitations (space) could be acting to control the final achene number and size into the capitulum.

Intra-flower competition in inflorescences have not been deeply studied. An unique and elegant example was given by Jeune and Barabé,[21] who explored with a mathematical approach the geometrical and biophysical rules that affect the spatial arrangement of inflorescence components in the Araceae. They compared the structure of the compact inflorescences of this family with a theoretical structure, where the average number of sides of floral primordia neighboring an *n*-sided primordium was determined by physical laws. They observed that a discrepancy between the measured shape and organization of those inflorescences and those predicted by theory was accounted for by the effect of physical constraint due to the proximal environment of developing primordia.

In the present study we examined the relative influences of capitulum architecture and whole plant resources as mechanisms for setting achene production in sunflower. So an accurate determination of the rate of development of the remaining achenes after performing the process of selective removal of flowers in various parts of the sunflower capitulum was performed.

Materials and Methods

Plant material

Seeds of the inbred line HA89-B were sown under field conditions in Pedro Luro, Argentina (39°26'S; 62°40'W). The soil of the experimental site is a Udic haplustol with sandy texture and low organic matter content (2.2%).[22] Plants were grown at a crop population density at flowering of 6 plants/m^2. Plot size was 5 rows × 15 m with an inter-row spacing of 0.70 m.

The crop was managed according to the recommended conventional agronomical practices.[23] Environmental conditions during crop growth kept soil water content above 50% of maximum soil available water. When necessary, soil water was supplemented by furrow irrigation. Nutrient deficiencies were prevented with pre-planting and pre-flowering fertilization with nitrogen (60 Kg N.ha^{-1}) applied as NO_3K. Weeds were controlled manually. Insect pests were not an important factor during the whole growing season.

During the study, mean air temperature (°C) and solar radiation (μmol.m^{-2}s^{-1}) were daily recorded with two-hour interval using a WS-GP1 Compact Automatic Weather Station (Delta-T Devices Ltd., Cambridge, UK). Maximum and minimum temperatures during the reproductive period as well as average daily incident radiation were between the range of optimum magnitudes for sunflower.[1] Table 1 summarizes the seasonal pattern shown by the environmental variables registered during the experimental period.

Flower removal experiments

Crop phenology was followed every two-three days from emergence to harvest maturity. Vegetative and reproductive development observations followed the Schneiter and Miller scale.[24] When plants reached first anthesis (FA; phenological stage R5.1)[24] 180 plants were labeled for experimentation.

Treatments started in these selected plants between late R7 and early R8.[24] At that time achene filling in the peripheral flowers had begun.[25] At R8 all flowers had reached full development within the inflorescence.

Four localized source/sink modifications treatments were performed: *outer flowers removed* (OR; Figure 1A), in which flowers in the outer third of the capitulum radius were removed, *inner flowers removed* (IR; Figure 1B), in which the inner third of flowers was removed. Control, in which inflorescences were not manipulated (Figure 1C) and *outer flowers removed + perimetral constraint* (OR+PC; Figure 1D). In this treatment, after the flowers of the outer third of the capitulum radius were removed, a semi-elastic ring of high density expanded polystyrene 2.5 cm thick was applied to partly restrain its radial expansion during achene growth and capitulum maturation. To retain the ring onto the receptacle, 4 stainless steel clips equally distributed on the receptacle surface were used (Figure 1D). During the period of capitulum maturation, rings were observed to be always kept in position. At the beginning of this last procedure capitulum diameter of selected plants averaged 9.3±1.3 cm.

The number of flowers removed in the treatment where outer flowers were removed was between 5 to 10% higher than that in the treatment where inner flowers were removed.

Achene dry mass determination

Starting from the treatment day, achenes from three capitulum sectors, external (ES), middle (MS) and internal (IS), each equal to 1/3 of the capitulum radius were harvested every 3 to 7 days up to harvest maturity (HM). At each harvest time, 10 achenes per plant were taken from 3 selected plants per plot that had not been sampled previously. Ovaries were

Table 1. Mean environmental data for three developmental crop phases. Crop phase A represents the 15-d interval centered on the mean date of full anthesis. Crop interval S-A is the time elapsed between sowing and 7 days before the mean date of full anthesis. A-PM is the interval between 7 days after full anthesis the date of PM.

Environmental factor	Plant developmental phase		
	S-A	A	A-PM
Max. temperature, °C	22.6	25.2	31.5
Min. temperature, °C	13.2	17.1	14.8
Daily incident radiation, MJ m^{-2}	22.3	23.2	21.7

A, anthesis; S, seeding time; PM, physiological maturity.

Table 2. Effect of floret removal on average dry mass per filled achene at harvest maturity and percentage of empty achenes.

Treatment, capitulum sector	Max. achene mass (mg) and empty achenes (%) at harvest maturity in each capitulum sector					
	External		Middle		Internal	
	Max. achene mass, mg	Empty achenes per sector, %	Max. achene mass, mg	Empty achenes per sector, %	Max. achene mass, mg	Empty achenes per sector, %
Control						
External	61.5 (3.3)^{a}	12.1^{a}	-	-	-	-
Middle	-	-	44.9 (2.6)^{a}	15.3^{a}	-	-
Internal	-	-	-	-	33.1 (2.7)a	23.0^{a}
Outer ring removed						
Middle	-	-	56.2 (3.1)^{b} [+27.6]	7.2^{b}	-	-
Internal	-	-	-	-	37.6 (2.9)b [+13.6]	19.7^{a}
Outer ring removed + PC						
Middle	-	-	49.8 (3.7)^{a} [+7.1]	11.9^{b}	-	-
Internal	-	-	-	-	34.0 (3.2)a [+2.7]	18.4^{b}
Inner ring removed						
External	67.7 (2.9)^{b} [+9.3]	13.2^{a}	-	-	-	-
Middle	-	-	53.6 (3.5)^{c} [+17.9]	12.7^{a}	-	-

Values in column with different letters are significantly different ($P<0.05$). PC, peripheral constraint. Values in parentheses ±1SE. Values in the same column followed by different letters are significantly different ($P<0.05$). Values in brackets = percentage of achene dry mass (mg) change against control values for the same capitulum sector.

kept in a cooler with ice and taken to the laboratory within 90 minutes of sampling. The achenes were dried at 70°C for at least 48 h before weighing. Achene dry mass (ADM) was then calculated.

After ripening the collected capitula were taken to the laboratory and the achenes were separated from treated and control inflorescences. All achenes were taken from each capitulum separately in three concentric zones, equal in width (external, middle and internal). Total number of empty achenes per sector was counted and ADM was then measured.

Statistical analysis

Time course of achene mass increase was estimated fitting a bilinear regression to the data using a nonlinear routine of the Kaleidagraph version 4.1 software (Synergy Software, Reading, Pennsylvania, USA). The initial straight line is defined by $p=a1+bX$ (for $X<c$) and the second straight line is defined by $p=a2$ (for $X>c$), where p is the ADM (mg), X represents time (days from R7) a1 is the intercept, b is the slope of the non-plateau section, (defines achene growth rate), a2 is the value of the plateau of the function (indicates the maximum ADM (mg) and c, the unknown breakpoint (*i.e.,* the timing of maximum ADM).[25,26]

All data were statistically analyzed by a two-way analysis of variance (ANOVA) using the Infostat statistical software package v. 2012.[27] The least significant difference (LSD) test was used to separate differences between capitulum sectors achene mass and their interactions.

Results

Flower removal affected achene mass after treatments of inner or outer achenes (Table 2; Figure 2). Achenes were heavier in those treatments in which internal or external flowers were removed in comparison with control ones (Table 2; Figure 2). Peripheral achenes of inflorescences where inner flowers were removed (Figure 1B) were significantly heavier (67.7±2.9 g; $P<0.05$) than the outermost achenes of inflorescences of control plants (61.3± 3.3 g; $P<0.05$; Table 2).

Floret removal of the outer third capitulum ring significantly improved achene mass in the middle and center (Table 2; Figure 2B). In treated plants, the percentage of empty achenes in the middle sector was lower (7.2% and 11.9%, Table 2), than in control plants (15.3%, Table 2) as well as in the middle sector (19.7% and 18.4% *vs*. 23.0%). For IR treatment, maximum ADM in the external third and middle sector was observed 16 and 17 days respectively after R7 (Figure 2B).

In the treatment when the inner third was removed (Figure 1B) final achene dry mass of the middle sector was attained 3.7 days later than in control plants (Figure 2A). On the other hand, in the treatment when the outer third was removed (Figure 1A) achenes of the middle sector attained its final dry mass 1.5 days earlier (Figure 2B). For OR treatment, maximum ADM in the internal third and middle sector was attained two days earlier than in control (Figure 2B).

Generally in al cases, except for the MS achenes in the IR treatments, the slope of the achene dry mass gain, was higher than in control (Figure 2; Table 2) indicating that the achenes were not only gaining higher mass compared with controls but also that this process was occurring faster. In the first case it may happen that the period of achene filling lengthens in the MS because there is no space limitations as a result of absent central achenes. In the second case, it can happen something similar but with an effect half controlled by the achenes of the MS. Also, as the ovaries of the IS may be in some cases smaller,[14] these achenes gain mass before and quickly reach their maximum mass.

In capitula with outer ring floret removal (OR+PC) a peripheral constraint, which in turn is mechanically equivalent to being under compression, significantly reduced the final ADM of achenes of the MS compared with those plants with the peripheral removed ring and no constraint (OR; Table 2). Nevertheless the final achene mass was significantly higher for the same capitulum sector in control plants (Figure 2B; Table 2).

Discussion

Following abundant evidence related with resource competition among achenes, independently of floral initiation order, appear to be that later developed achenes, affect the size of achenes produced earlier, so achenes from the IR treatment should be heavier than OS achenes in control inflorescences.

But, on the other hand, spatial effects where position also affects achene mass, suggest that the OS achenes from the IR treatment should be heavier than the OS achenes from the OR treatment, as the latter are in a relative inner position compared to the IR ones.

In this work ovary removal overall produced an increase in the size of the remaining achenes, suggesting competition for resources as one of the causes of the observed position effect. But on the other hand, the experimental manipulations of the source/sink ratio not only demonstrate that resource limitation is a major determinant of achene mass, in the capitulum,[16,20] but that can mask a spatial effect on the development capacity of the remaining achenes. In this sense spatial issues should also be considered.

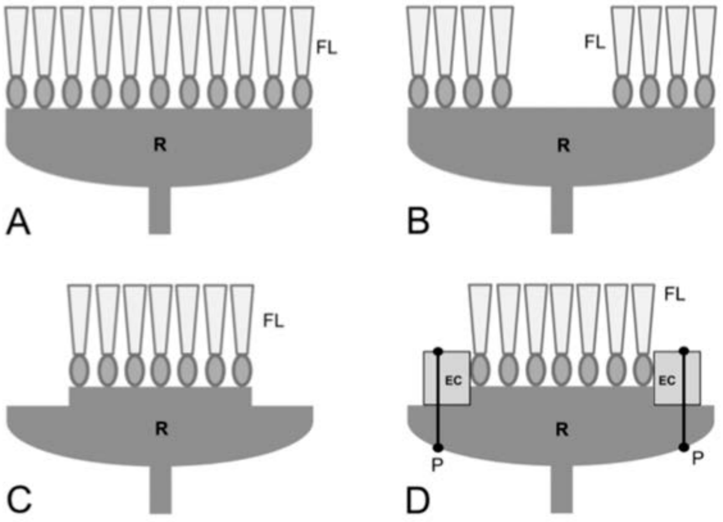

Figure 1. Schematic representation of the four treatments control (A), *inner flowers removed* (IR; B), *outer flowers removed,* (OR; C) and *outer flowers removed plus external constraint* (OR+PC; D), applied to capitula of sunflower in the experimental manipulation of flower competition. EC: Styrofoam ring to apply an artificial external constraint to the middle ovaries; R: receptacle; FL: florets; P: fixing pins of the EC onto the receptacle tissue.

In fact, in the experiment where a physical restriction of capitulum radial expansion was placed after removal of the OS ovaries (Figure 1D), resulted in lesser growth of the IS achenes than in the case of the removal of the OS ovaries without the external constraint (Table 2; Figure 2). Therefore, we should not ignore physical effect imposed by internal forces of expansion/compression of developing achenes in a whole capitulum.

In Figure 3 a conceptual expansion model in a post fertilized capitulum is presented. In this model an interpretation of how the forces of achene expansion and radial receptacle expansion would interact on a particular growing achene (Figure 3A).

We can infer that the dynamics of achene growth will result from the interrelation of the physiological component (assimilate input) plus two main geometrical components: the physical expansion of the receptacle surface (Figure 3B) and the rate at which achene growth is occupying this surface (Figure 3C).

The rate of achene growth will quickly increase as the receptacle surface cease its expansion growth probably produced by decrease competition for nutrients or growth substances by the proximal achenes, now engaged in active growth.

Exploratory work on determination of receptacle expansion after R8 using landmarks showed that radius growth, expansion rate diminishes from the center towards the periphery (Hernández, unpublished) an inverse finding previously reported,[28] in the interval in FS5 to 6 when the receptacle meristem is differentiating into floret primordia. In there, he found that capitulum expansion rate was higher at the periphery and slower or absent at the receptacle center. So if the disc center tends to grow rapidly (have excess surface)[29,30] compared to the margins then the margins will respond, passively, with buckling,[31,32] and it would necessarily result in the formation of a the rounded downwards rim observed in mature capitula after R8.[33]

Conclusions

It is concluded that achene size is highly controlled by restrictions in space. Achenes that develop in the (external) proximal position of the capitulum have an advantage over those that develop in the (internal) distal portion thereof. Being the first to start developing and the most external, the radial expansion of the capitulum during grain filling and maturation diminishes the pressures between neighboring achenes but are higher as one moves towards the capitulum center. So, when more we move towards the center, significant coun-

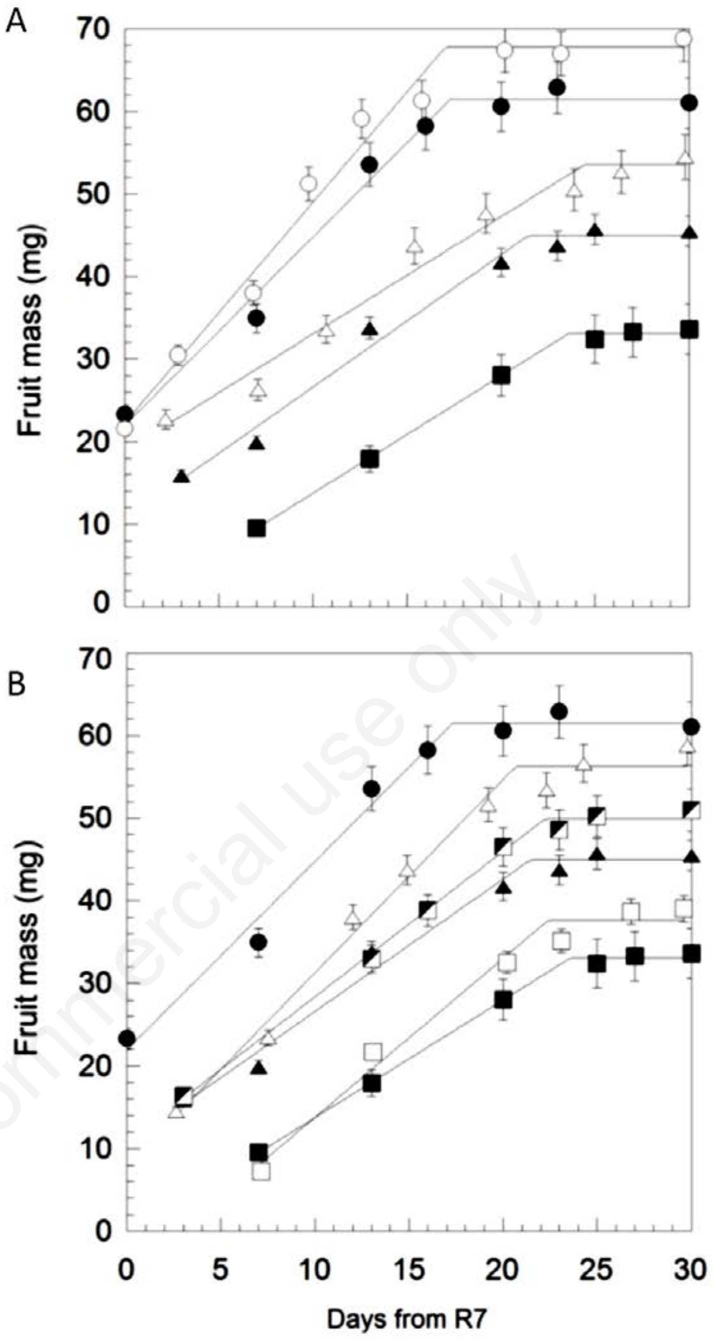

Figure 2. Evolution of achene dry mass (ADM) from R7 up to harvest maturity for the controls plants (closed symbols) and treated plants (open symbols) respectively. A) Control plants vs. external ring of florets removed (OR) and external ring removed plus a peripheral constraint (OR+PC). B) Control plants vs. internal ring of florets removed (IR). (●, ○) external achenes; (▲, △) middle achenes; (■, □) internal achenes, (◩) middle achenes when an artificial peripheral constraint was applied. For this last treatment data for the internal achene mass is not shown. Vertical bars: ±1 SE. Fitted values are shown in the following equations:

● $y=21.96+2.28x$ $R^2=0.996$
○ $y=22.29+2.67x$ $R^2=0.993$
▲ $y=10.57+1.60x$ $R^2=0.992$
△ $y=18.84+1.42x$ $R^2=0.974$
■ $y=-0.46+1.42x$ $R^2=0.989$
□ $y=-5.45+1.94x$ $R^2=0.987$
◩ $y=10.79+1.76x$ $R^2=0.979$

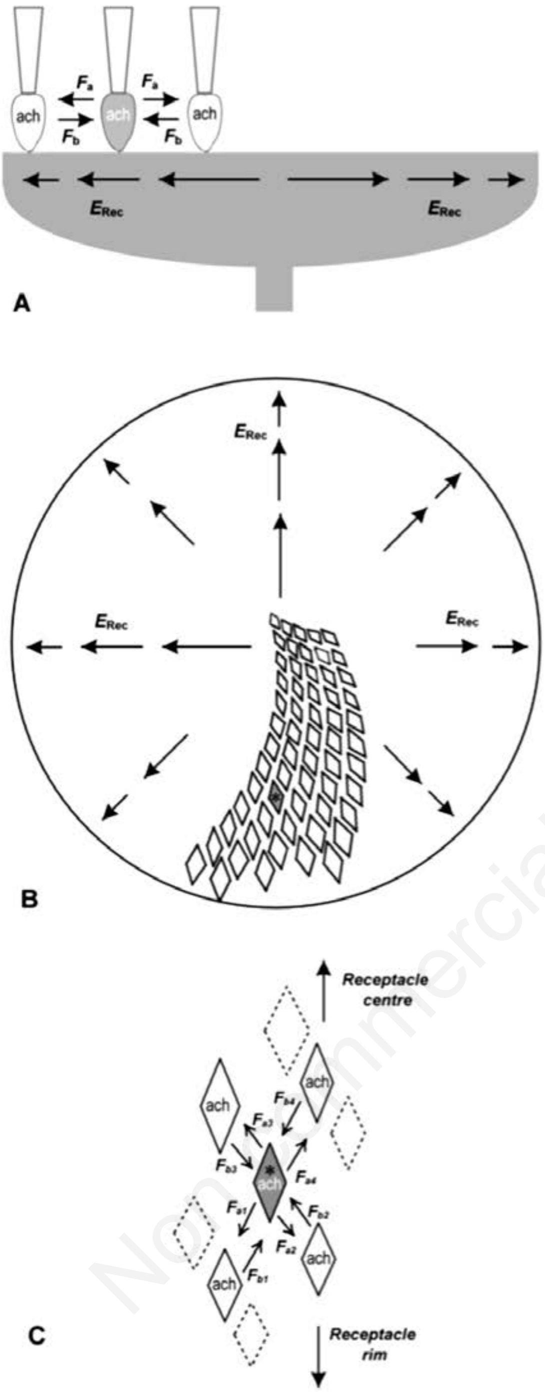

Figure 3. Schematic representation of the conceptual model of receptacle radial expansion, achene growth spatial competition. Simplified model of achene growth from the point of view of forces interacting during the development of a given achene on the receptacle surface. While the whole receptacle is radially expanding (A, B) each growing achene confronts with the competition for space with its neighbors (C). ach: achene; F_{a1-4}: expansion forces exerted by a particular achene. F_{b1-4}: expansion forces exerted by neighboring achenes. Regarding the neighboring achenes developmental age, because its relative position in the receptacle (C), then $F_{b1}>F_{b2}>F_{b3}>F_{b4}$. For simplification of the model, F_{a1-4} is equal in all directions. $E_{rec:}$ receptacle radial expansion. Different arrow lenght represents the expansion rate magnitude gradient into the receptacle after R8, as observed by Hernández (unpublished).

tervailing forces would begin to appear that would multiply from the outside in.

The results shown here constitute important experimental evidence supporting both resource competition and physical/spatial effects on achene size reduction within the sunflower inflorescence. The results also suggest that, similarly, the physical constraints during the capitulum development may limit the size/mass of internal achenes compared with the external ones, but the proximate mechanism of architectural effects remains uncertain.

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