

Is the abaxial palisade parenchyma in phyllaries of the sunflower (*Helianthus annuus* L.) capitulum a missing trait in modern genotypes?

¿Es el parénquima en empalizada abaxial en las filarias del capítulo del girasol (*Helianthus annuus* L.) un rasgo ausente en los genotipos modernos?

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Abstract. The involucre bracts (IB or phyllaries) of the sunflower (*Helianthus annuus* L.) capitulum constitute an example of photosynthesizing organs that contribute to the photosynthesis budget during the generation of crop yield. The anatomy of IB was analyzed in two domesticated primitive sunflower genotypes, Havasupai and Hopi, in the sunflower line HA89B, in the wild *Helianthus annuus* ssp. *annuus*, in the sunflower male-fertile line R013 and in the commercial hybrid DKOP3845. Stomata and trichomes were counted on the adaxial and abaxial epidermis. In all cases, the IB showed a one-layered adaxial and abaxial epidermis, secretory ducts and parenchymatic cells with abundant chloroplast. The vascular system was similar to that of the nomophylls; however, their bundles were smaller, with an abaxial surface showing abundance of glandular and non-glandular trichomes and stomata. IB of Havasupai, Hopi and the male-fertile line strain showed higher number of adaxial hypodermic strata than those of HA89B, DKOP3845 and wild sunflower (2-3 vs. 1), and one mesophyll with inverted polarity with respect to a foliage leaf: the presence of a spongy parenchyma on the adaxial side was observed with a rudimentary palisade parenchyma on the abaxial side. Stomatal density of the IB was significantly higher in Hopi and Havasupai than in HA89B and DKOP3845, with values ranging from 132 to 156 vs 73 to 110 stomata/mm², respectively. Like the modern commercial hybrid, the IB of male-fertile line showed lower stomatal density (83 stomata/mm²) and scarce abaxial trichomes. The anatomical studies of phyllaries in Asteraceae remain relatively poor in the literature. From the functional point of view, sunflower breeding produced undesired changes in the IB anatomy. Future studies for comparing the physiological (photosynthesis and respiration) and biochemical activities of the RuBisCO among the IB of the primitive (Havasupai and Hopi), male-fertile lines and modern sunflower genotypes will help to define the magnitude of their importance as a morphological trait to be considered in future plans of sunflower breeding.

Keywords: Inverted anatomy; Involucre bracts; Modern genotypes; Primitive genotypes; Sunflower.

Resumen. Las brácteas involucrales (BI o filarias) del capítulo de girasol (*Helianthus annuus* L.) constituyen un ejemplo de órganos fotosintéticos que contribuyen al balance de la fotosíntesis durante la generación del rendimiento del cultivo. Se estudió la anatomía de las BI de girasol en dos genotipos domesticados primitivos, Havasupai y Hopi, en la línea HA89B, en el girasol silvestre *Helianthus annuus* ssp. *annuus*, en la línea androfértil R013 y en el híbrido comercial DKOP 3845. Se observaron estomas y tricomas en las epidermis adaxial y abaxial. Todas las BI mostraron una sola capa de epidermis adaxial y abaxial, conductos secretores y células parenquimáticas con abundantes cloroplastos. El sistema vascular fue semejante al foliar; pero con haces más pequeños, y la superficie abaxial presentó mayor abundancia de tricomas glandulares y no glandulares y estomas. Las BI de Havasupai, Hopi y la línea androfértil R013 presentaron un mayor número de estratos hipodérmicos adaxiales que HA89B, DKOP 3845 y el girasol silvestre (2-3 vs. 1), y un mesófilo con la polaridad invertida con respecto a la hoja: el parénquima esponjoso se encontró en la cara adaxial, y el rudimento de parénquima en empalizada en la cara abaxial. La densidad estomática de las BI fue significativamente mayor en Hopi y Havasupai que en HA89B y DKOP3845, con valores de 132, 156, 73 y 110 estomas/mm², respectivamente. Al igual que el genotipo comercial, las BI de la línea androfértil mostraron una baja densidad estomática (83 estomas/mm²) y escasos tricomas en la cara abaxial. Los estudios anatómicos de las filarias en Asteráceas siguen siendo relativamente pobres en la literatura. Desde el punto de vista funcional, el mejoramiento en girasol ha producido cambios no deseados en la anatomía de las BI. Futuros estudios que comparan los efectos fisiológicos (fotosíntesis y respiración) y de la actividad bioquímica de la RuBisCO entre las BI de los genotipos primitivos (Havasupai y Hopi), líneas androfértiles y genotipos modernos de girasol, ayudarán a definir la magnitud de su importancia como un rasgo morfológico a considerar en futuros planes de mejoramiento del cultivo.

Palabras clave: Anatomía invertida; Brácteas involucrales; Genotipos modernos; Genotipos primitivos; Girasol.

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INTRODUCTION

Leaf photosynthesis is one of the main factors of source strength, and plays an important role in determining crop yield. Given the substantial variations in leaf anatomical features among and within plant species, a possibility exists to modify leaf anatomy to improve photosynthetic efficiency (Long et al., 2006; Hall & Richards, 2013).

Green tissues adjacent to the main sinks at the fruit filling stage (i.e., bracts and upper leaves) play a significant role in providing photoassimilates to these sinks. Contribution to CO₂ fixation by non-foliar organs, such as reproductive structures (Weiss et al., 1988; Blanke & Lenz, 1989), stem tissues (Nilsen, 1995; Pfanz & Aschan, 2001) and even roots (Kitaya et al., 2002) has been demonstrated by several authors.

The involucre bracts (IB), botanically defined as phyllaries, of the sunflower (*Helianthus annuus* L.) capitulum constitute an example of photosynthesizing organs that contribute to the photosynthesis budget during the generation of crop yield. Laxman & Srivastava (2000b) compared the IB photosynthetic and respiratory activities with those of nomophylls in two genotypes of sunflower at different growth stages. During ontogeny, the bract net Ribulose Biphosphate Carboxylase (RuBisCO) activity increased gradually until seed filling. But the RuBisCO activity of IB was 10 to 14 times lower than in the nomophylls. The lower photosynthetic rate of bracts relative to nomophylls was due to lower stomatal conductance (Laxman & Srivastava, 2000a) and low chlorophyll concentration due to the absence of palisade cells (Laxman & Srivastava, 2000b). They also found that PEP carboxylase and NADP-malate dehydrogenase activity in bracts was higher than that in nomophylls, which indicated the possibility of involvement in refixation of respiratory released carbon (Laxman & Srivastava, 2000b).

The contribution of IB to the sunflower inflorescence carbon balance could also become relevant under water stress (Hu et al., 2014). IB in sunflower have the appearance of drought tolerant organs with xerophyte morphology. This carbon contribution has been observed in cotton (Hu et al., 2014) and in the awns of wheat (Evans et al., 1972). Flemmer et al. (personal communication, Departamento de Agronomía, Universidad Nacional de Sur, Argentina) observed an increased presence of sclerenchyma in the IB of safflower (*Carthamus tinctorius* L.). This feature corresponds to the situation in most land plants, especially those growing in arid environments.

The aim of the present work was to identify morphological changes occurring in the sunflower phyllaries as a result of its genetic improvement, in order to either consider or discard their incorporation as a morphological feature that may be improved in future breeding programs.

MATERIALS AND METHODS

Two domesticated primitive sunflower genotypes Havasupai and Hopi, the line HA89B, the wild sunflower (*Helianthus annuus* ssp. *annuus*), the male-fertile line R013 (Manfredi Experimental Station-INTA) and the commercial hybrid DKOP3845 (Monsanto, Argentina) were grown at the experimental field of the Departamento de Agronomía, Universidad Nacional del Sur, Bahía Blanca, Argentina (38° 45' Lat. S; 62° 11' Long. W). The soil was a Typic Ustipsamment (Soil Survey Staff, 1999). At growth stage V4 (plants with 4 nomophylls; Schneider & Miller, 1981), plant density was adjusted to 5.6 plants/m² except for the wild line where plant density was adjusted to 3.3 plants/m². At sowing time and at anthesis (stage R5) plant were fertilized with potassium nitrate (NO₃K, 15% N), at a rate of 60 kg N/ha. For multi headed plants (the wild sunflower and the male-fertile line R013) the time of anthesis was considered for the main capitulum. Soil water content was maintained at optimum levels by drip irrigation during the whole crop cycle. Weeds were adequately controlled manually. Plague or disease control was not necessary.

For histological analysis and quantification of stomata and trichomes in the abaxial and adaxial epidermis, six IB from the external whorl of the capitulum of three plants per genotype were collected at stage R8 (Schneider & Miller, 1981). In the multi headed plants the main capitulum was considered for IB sampling.

For histological study, segments of the medium portion of three IB per plant were placed in vials containing FAA fixative (formalin-acetic acid-alcohol-water, 10:5:50:35; Ruzin, 1999). The tissue segments were processed following conventional histological techniques (Ruzin, 1999), embedded in Paraplast (Leica) and cut at 8-10 mm with a rotary microtome. They were then stained with safranin-fast green (Ruzin, 1999), observed and photographed using a Nikon Labophot-2 microscope equipped with a Nikon Coolpix 4500 digital camera.

To observe and quantify stomata and trichomes, molds of the abaxial and adaxial epidermis of three IB per plant were taken using the technique described by Hernández & Green (1993). Shortly, a selected area on each side of the bract was covered with an hydrophilic polyvinyl siloxane impression material (Mirror 3, Kerr Manufacturing Co., Romulus, Mich, USA). Once polymerized, the material was removed with tweezers and painted with a layer of colorless synthetic enamel paint. The enamel copies were dry mounted on microscope slides and observed and photographed under a microscope (Nikon Labophot-2) at ×400 magnification. With the digital images and the ImageJ software (Rasband, 2011), three areas of 0.1 mm² per image were selected and the stomata counted, obtaining an estimation of stomatal density (number of stomata/mm² IB). Data obtained were subjected to analysis of

variance and the difference among measurements was compared using the Tukey test with $P \leq 0.05$ (Di Rienzo et al., 2010).

RESULTS

All the IB analyzed showed the same, including nomophylls, an adaxial and abaxial unstratified epidermis, secretory ducts, parenchymatic cells with abundant chloroplasts, a vascular system with a single series of vascular collateral bundles, which were smaller than the foliar ones, and an abaxial surface with a higher abundance of glandular and non-glandular trichomes and stomata (Fig.1).

Stomata were scattered on the abaxial side and were especially abundant on the exposed distal portion of the phyllaries. Guard cells tend to be oriented parallel to the long axis of the bracts, but many were transverse or oblique. The stomatal apparatus in the IB was almost identical to that found in the nomophylls.

In Hopi, Havasupai, wild sunflower and the male-fertile line, some columnar cells or square cells, on the abaxial side of the IB, with many chloroplasts may be designated as a rudimentary palisade parenchyma (Fig. 1A, B, C and F, respectively). These IB showed dorsiventral heterogeneous mesophyll with inverted polarity with respect to nomophylls, so the spongy parenchyma was on the adaxial surface and the rudimentary palisade parenchyma was on the abaxial side (Fig. 1). These genotypes, except the wild sunflower, showed IB with a multistratified adaxial hypodermis (Table 1).

Higher stomatal and trichome density was observed in Hopi and Havasupai than in HA89B and DKOP3845, with values ranging from 132 to 156 vs. 73 to 110 stomata/mm², respectively (Table 1). Like the commercial genotype, the IB of the male-fertile line showed lower stomatal density (83 stomata/mm²) and scarce abaxial trichomes (Table 1).

Likewise stomatal density in the IB of all genotypes evaluated was significantly lower than values reported for nomophylls by Rawson & Craven (1975), i.e. 73-156 vs. 276 stomata/mm² respectively (Table 1) and no stomata were found in the adaxial side in phyllaries of any studied genotype (Fig. 1).

DISCUSSION AND CONCLUSIONS

The most notorious anatomical character observed in the phyllaries studied here was a presence, in the primitive strains and multi headed genotypes, of a rudimentary palisade parenchyma in the abaxial side (Fig. 1). The IB of Hopi and Havasupai showed a 2-3 layered adaxial hypodermis (Table 1), and an heterogeneous mesophyll composed of spongy and palisade parenchymas (Fig. 1A and B respectively). The wild sunflower showed IB with an one-layered adaxial hypodermis, and the male-fertile line showed IB with a 3 layered adaxial hypodermis (Table 1). Also both showed a heterogeneous mesophyll (Fig. 1C and F respectively). Apparently in these *Helianthus* genotypes the mesophyll was differentiated into palisade parenchyma in light-exposed regions (abaxial side) and spongy parenchyma in shaded parts of the leaf anatomy (adaxial side).

As observed in Hopi, Havasupai, male-fertile line and wild sunflower, this kind of anatomical tissue arrangement in the leaf mesophyll is often referred to as "adaxialized leaf" (McConnell & Barton, 1998; Johnson et al., 2005; Tian et al., 2007). Napp-Zinn (1973) described it for bracts of *Peperomia* adaxialized anatomy. A particular case is also described on leaves of two varieties of the variegated croton (*Codiaeum variegatum*) that exhibited both normal and inverse unifaciality in a single leaf (Baum, 1952) and it was explained as a variation of the expansion domain of a single adaxializing

Table 1. Morphological characters of involucral bracts in the sunflower genotypes studied in this work.

Tabla 1. Características morfológicas de las brácteas involucrales en los genotipos de girasol estudiados en este trabajo.

Sunflower genotype	Morphological characters					
	Adaxial hypodermal layers (Fig.1)	Abaxial hypodermal layers (Fig.1)	Adaxial spongy parenchyma	Abaxial palisade parenchyma	Abaxial stomata* (No/mm ²)	Abaxial trichome occurrence
Line HA89B	1	1-2	YES	NO	73 a	Scarce
Male-fertile line R013	3	0-1	YES	YES (Rudiment)	83 a	Scarce
Hybrid DKOP3845	1	1	YES	NO	110 b	Scarce
Wild sunflower	1	3	YES	YES (Rudiment)	129 bc	Ample
Hopi	3	2	YES	YES (Rudiment)	132 c	Ample
Havasupai	2	3-4	YES	YES (Rudiment)	156 d	Ample

* In a column, means followed by different letters are significantly different (LSD test, $P \leq 0.05$).

* En una columna, los valores seguidos por letras diferentes difieren significativamente (prueba LSD, $P \leq 0,05$).

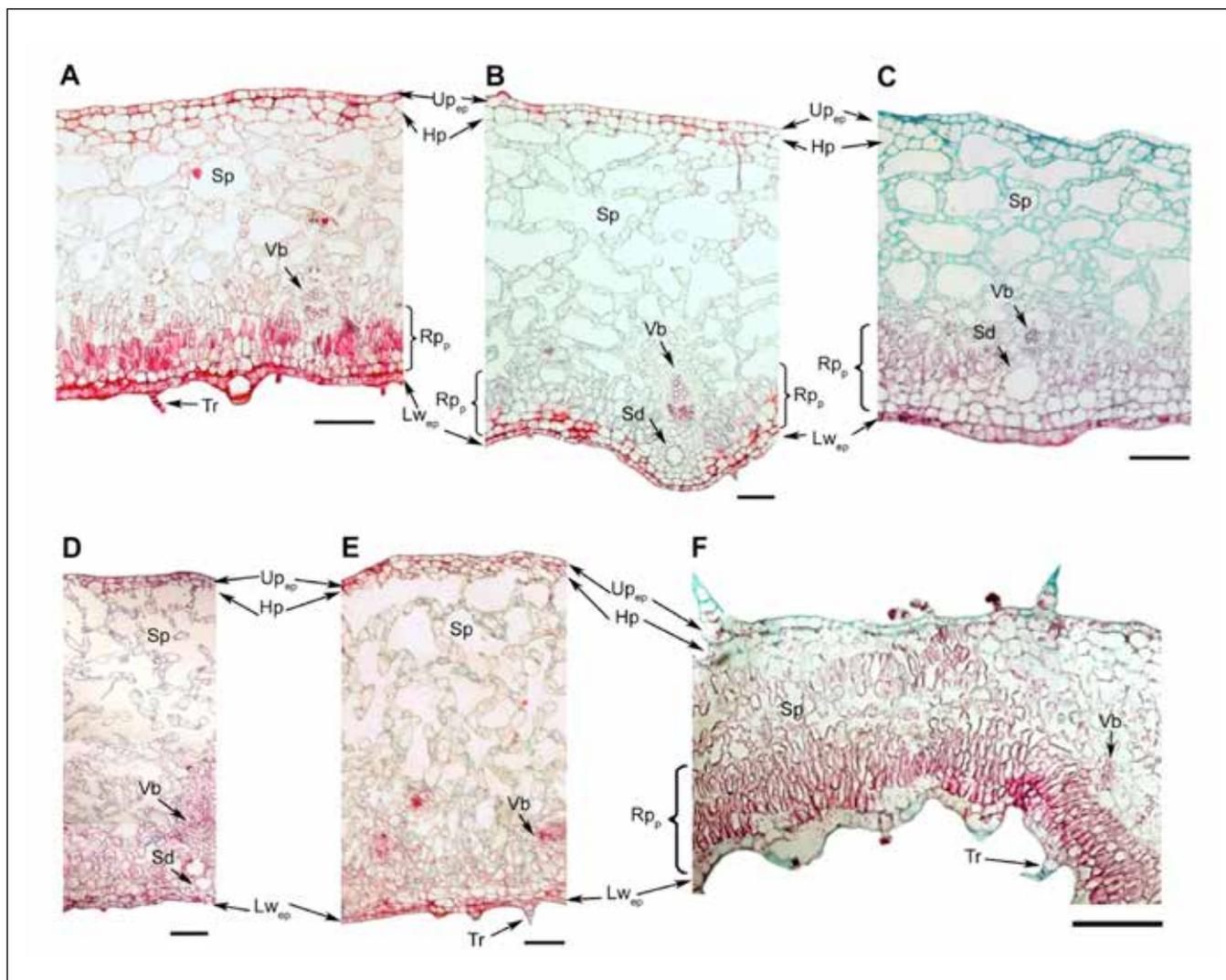


Fig. 1. Involucral bracts cross sections of Hopi (A), Havasupai (B), wild sunflower (*Helianthus annuus* ssp. *annuus*, C), DKOP3845 (D), HA89B (E) and a male-fertile line R013 (F). Hp: hypodermis, Lwep: lower epidermis, Rpp: rudimentary palisade parenchyma, Sd: secretory duct, Sp: spongy parenchyma, Tr: trichoma, Upep: upper epidermis, Vb: vascular bundle. Scale: 200 μ m.

Fig. 1. Sección transversal de las brácteas involucrales de Hopi (A), Havasupai (B), girasol silvestre (*Helianthus annuus* ssp. *annuus*, C), DKOP3845 (D), HA89B (E) y la línea androfértil R013 (F). Hp: hipodermis, Lwep: epidermis abaxial, Rpp: parénquima en empalizada rudimentario, Sd: conducto secretor, Sp: parénquima esponjoso, Tr: tricoma, Upep: epidermis adaxial, Vb: haz vascular. Escala: 200 μ m.

factor (Gleissberg, 2000). Church (1914) noted that palisade tissue in pine cone bracts occurred in regions of the female cone that were exposed towards sunlight.

This phenomenon of “reversed anatomy” has also been reported for vegetative organs in other species such as nomophylls of mutant *Arabidopsis thaliana* (McConnell & Barton, 1998), *Zea mays* Rolled leaf1 (*Rld1*, Nelson et al., 2002) or Pichi (*Fabiana imbricata*) leaves exposed to direct sunlight or shade (Cosa et al., 2012).

Western red cedar (*Thuja plicata*) and Chinese thuja (*Platycladus orientalis*) display anatomical differences which are mainly influenced by different exposures to light (Dörken, 2013). The median leaves inserted at the exposed upper side of the shoot show

“adaxial leaf anatomy” by a predominant development of palisade parenchyma. However, median leaves inserted at the shaded lower side of the shoot show “abaxial leaf anatomy” and an important development of spongy parenchyma (Dörken, 2013).

Plants can then respond to different levels of sunlight exposure changing internal leaf anatomy such as the amount and distribution of palisade and spongy mesophyll tissue. Many of these anatomical changes have been associated with the regulation of light and CO₂ profiles inside the leaves (Parkhurst & Mott, 1990; Parkhurst, 1994; Terashima & Hikosaka, 1995; Vogelmann et al., 1996). It has also been proposed that these structural changes have evolved in concert with properties of leaf orientation, maximizing photosynthesis (Smith et al., 1997; 1998).

The presence of palisade parenchyma on the side of the IB of primitive and wild genotypes exposed to light (abaxial side) could present an important physiological advantage in this photosynthetically active organ, with respect to the homogeneous mesophyll present in the IB of modern hybrids. Also the higher density of the stomata in the IB of primitive genotypes could facilitate an increased stomatal conductance, as compared to the IB of modern genotypes that have lower stomatal density (Laxman & Srivastava, 2000a).

It is known that the specification and development of leaf dorso-ventral polarity is regulated by adaxializing transcription factors (Bowman et al., 2002) and microRNAs that act in the abaxial domain of the leaf primordium (Emery et al., 2003; Juarez et al., 2004; Kidner & Martienssen, 2004).

The molecular identity of the adaxialization signal is not fully known. Nevertheless, analysis of the *Arabidopsis* mutant enlarged *fil* expression domain1 (*enf1*) (Toyokura et al., 2011) suggest that a metabolite in the GABA metabolic pathway is involved in establishment of the adaxial domain.

A number of candidate genes that are involved in these processes have been identified (Mathur, 2004; Panteris & Galatis, 2005). The molecular mechanisms controlling leaf morphology traits that could act changing the photosynthetic rate and/or leaf anatomy are still unknown (Tholen et al., 2012).

The IB of the domesticated primitive genotypes shows a mesophyll with an inverted anatomy, possibly because of the response to direct exposure to solar radiation received by the abaxial surface. Absence of palisade parenchyma during the anatomical development in the IB of modern sunflower may reflect loss of that trait during human selection, or may be due to a different cause or set of causes not studied in this work.

We cannot assure with our findings if the presence of a palisade parenchyma could improve photosynthesis in the phyllaries, and subsequently if this could enhance the carbon balance in the receptacle. But it is evident that this anatomical trait is present in this species, and should be a challenge to introgress it in modern genotypes without losing the yield efficiency already achieved in the actual hybrids.

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