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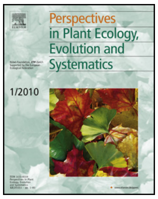

Research article

Diversity, evolution, and function of stomata bearing structures in *Cuscuta* (dodders, Convolvulaceae): From extrafloral nectar secretion to transpiration in arid conditions

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ABSTRACT

Cuscuta includes ca. 200 species of functionally holoparasitic plants grouped in four subgenera: *Monogynella*, *Cuscuta*, *Pachystigma*, and *Grammica*. Multicellular structures with stomata in *Cuscuta* are represented by extrafloral nectaries (ENs), reported from the stems of one *Monogynella* species, and stomatiferous protuberances (SPs), which are non-secretory. These latter structures had been noted on the stems of three *Grammica* species more than a century ago but entirely forgotten until recently when similar, non-secretory SPs were reported on the flowers of several new *Grammica* species. Here we study for the first time: (1) the extent of occurrence, diversity and evolution of secretory (ENs) and non-secretory (SPs) multicellular structures in *Cuscuta*, and (2) the function of SPs. We undertook a character evolution study of ENs and SPs on the stems and flowers of 136 *Cuscuta* taxa, and examined the structure/ultrastructure of SPs. ENs are inferred as primitive and characterize subg. *Monogynella*. SPs are derived in the remaining subgenera; they are ubiquitous on the flowers of *Cuscuta* and *Pachystigma*, but absent on their stems. Subgenus *Grammica* species develop two functional types of stems during their life cycle: vegetative, exploratory stems with very low stomatal densities (and no SPs), and reproductive, haustorial stems with numerous SPs. Moreover, 24 species from nine clades of subg. *Grammica* have evolved morphologically diverse floral SPs with systematic significance. To preliminarily ascertain SP function, we determined in the field the water uptake of *Tithonia tubiformis* plants parasitized or not by *Cuscuta costaricensis*, a species with both stem and floral SPs, and the stomatal conductance of dodder stems and flowers, as well as host leaves. Water uptake of parasitized hosts was significantly higher compared to non-parasitized plants, even after host leaves were removed, both during the day and night. The increased water uptake of parasitized hosts and stomatal conductance values suggest a transpiration role for the SPs, which is also confirmed by their lacunar structure. *Grammica* species with floral SPs grow in arid areas or characterized by a pronounced dry season during flowering/fruiting, which suggests that SPs may have evolved to stimulate the host water uptake during these phenophases.

Introduction

Cuscuta (dodders) includes nearly 200 species of obligate stem parasites classified in four subgenera (García et al., 2014; Costea et al., 2015): *Monogynella* (mostly Eurasia, 15 sp.), *Pachystigma* (S. Africa, five sp.), *Cuscuta* (mostly Eurasia, 21 sp.), and *Grammica* (mostly the New World, 154 sp.). Approximately 15–20 *Cuscuta* sp. worldwide are agricultural and horticultural pests (Dawson et al., 1994; Costea and Tardif, 2006), and in most countries control and quarantine measures target the genus as a whole, ignoring the fact that more species may be endangered or even threatened with extinction (Costea and Stefanović, 2009).

Dodders exhibit a broad range of plastome reductions (reviewed by Braukmann et al., 2013) but even the most active photosynthetic species belonging to subg. *Monogynella* derive 99.5% of their carbon from their hosts and can be considered functionally holoparasitic (Jeschke et al., 1994b; Hibberd et al., 1998). As in other holoparasitic plants (Kuijt, 1969; Heide-Jørgensen, 2008), dodders have

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undergone drastic reductions of their vegetative organs, which has limited the morphological characters available for systematics to their flowers and fruits (Engelmann, 1859; Yuncker, 1932).

Extrafloral nectaries (EN) that release a solution of sugars via stomata were reported by Schaffner (1979) on the stems of *Cuscuta reflexa*, a species that belongs to subg. *Monogynella*, the first derived infrageneric lineage of *Cuscuta* (García et al., 2014). However, relatively similar morphologically but non-secretory multicellular structures termed "protubérances stomatifere" or stomatiferous protuberances (SPs) had been briefly observed almost eight decades earlier by Mirande (1901) on the stem epidermis of three species of subg. *Grammica* (*Cuscuta gronovii*, *Cuscuta chinensis*, and *Cuscuta americana*). Subsequent authors, however, found very low stem stomatal densities and transpiration rates for various subg. *Grammica* species (Yuncker, 1943; Patel and Inamdar, 1971; reviewed by Dawson et al., 1994), and Mirande's observations fell into oblivion. To complicate things further, non-secretory multicellular structures with stomata, similar morphologically to Mirande's stem SPs, were recently described on the flowers of several new species that belong to subg. *Grammica* (Costea and Stefanović, 2009; Costea et al., 2011a,b, 2013).

In this study we examine for the first time the extent of occurrence, diversity and evolution of secretory (ENs) and non-secretory (SPs) multicellular structures in *Cuscuta*, as well as the function of SPs. To generate a workable hypothesis for the latter, since 2006 we have observed in the field various *Grammica* species with SPs both on their stems and flowers (e.g., *Cuscuta bonafortunae*, *Cuscuta chapalana*, *Cuscuta costaricensis*, *Cuscuta cotijana*, *Cuscuta ortegana*, and *Cuscuta strobilacea*) to confirm that SPs do not discharge a fluid (e.g., like extrafloral nectaries or hydathodes). Since we did not detect a fluid release during the day or night in any of the species (Costea, unpublished), the most likely explanation is that SPs lose water vapor through their stomata (transpiration), a hypothesis that will be preliminarily tested in the field by this study. This would be particularly intriguing because, in general, holoparasitic plants possess low stoma densities and very reduced water loss (Raven, 1983; Seel et al., 1992; Dawson et al., 1994; Ehleringer and Marshall, 1995; Heide-Jørgensen, 2008). If the transpiration role is confirmed, it would also be interesting to explore some of the ecophysiological implications because all the recently described species with floral SPs are apparently confined to arid areas (Costea and Stefanović, 2009; Costea et al., 2011a,b, 2013), and a water loss in such conditions would be more than unusual.

Thus the main objectives of this study are to: (1) Bring back into attention Mirande's forgotten stomatiferous protuberances (SPs) and study for the first time their morphology, structure, and ultrastructure. (2) Comparatively survey the morphological diversity of ENs and SPs in *Cuscuta* using a comprehensive species sampling. (3) Determine evolutionary trends and explore the systematic significance of EN and SPs characters. (4) Preliminarily test in the field the hypothesis that stomata of SPs have a transpiration role. (5) Show that species with floral SPs are associated with a low precipitation regime during flowering/fruiting and discuss this finding.

Material and methods

Comparative morphological diversity of multicellular protuberances

Multicellular protuberances, ENs and SPs, on stems and flowers were examined in 136 *Cuscuta* taxa (122 species and 14 varieties; Table S1). We analyzed the same herbarium vouchers cited in three recent character evolution studies of *Cuscuta* (see the >500 herbarium vouchers cited in Welsh et al., 2010; Wright et al., 2011, 2012). In addition, for the recently described species, *Cuscuta insolita* and *Cuscuta iguanella*, we analyzed the herbarium specimens cited in Costea et al. (2013). Stem fragments and flowers removed from herbarium specimens were rehydrated in 50% ethanol (Riviere et al., 2013), examined with a Nikon SMZ1500 stereomicroscope, and imaged using Pax-it 7.5 software and a PaxCam Arc digital camera (MIS Inc. 2014, Villa Park, IL). For the micromorphological observations, rehydrated stem fragments, calyces and corollas were subjected to a hexamethyldisilazane (HMDS) treatment as an alternative to critical point drying (Wright et al., 2011). Samples were sputter-coated with 30 nm of gold using Emitech K 550 (Emitech, Ltd. Ashfort, UK). Imaging was done with a Hitachi SU-1510 variable pressure SEM at 3 kV. Thousands of morphology images for all the species have been uploaded in the Digital Atlas of *Cuscuta* (Costea, 2007-onward).

Character evolution

Eight characters were defined (Table 1) after a survey of the multicellular protuberances with stomata in the entire genus (136 taxa; Table S1). Character states were mapped onto the recent genus phylogeny based on *rbcL* and nrLSU sequences (García et al., 2014). Analysis of character polarity in *Cuscuta* using formal outgroup analysis is hindered by the unresolved position of *Cuscuta* in Convolvulaceae (Stefanović and Olmstead, 2004). Thus, to reconstruct ancestral character states in *Cuscuta* we analyzed the distribution of character states in-group (Welsh et al., 2010; Wright et al., 2011; Riviere et al., 2013). Adding putative outgroup Convolvulaceae and coding them with a different character state than the in-group *Cuscuta* (García et al., 2014) produced similar results (results not shown). Scenarios of character evolution were analyzed using the parsimony reconstruction method implemented in Mesquite 2.75 (Maddison and Maddison, 2011). Two qualitative, non-polymorphic characters (types of multicellular protuberances present on the stems and flowers; Table 1) were also analyzed with the likelihood reconstruction method provided by the same software. Markov *k*-state 1 parameter model (MK1) model of evolution was used; in the parsimony reconstruction, character-state changes were treated as unordered.

The correlation between the presence/absence of SPs on the calyx and corolla was examined using Pagel's (1994) method implemented in Mesquite. To test for a possible association between the presence of floral SPs and a dry climate we determined for all 136 taxa taxa two average precipitation values: annual and during the three months of maximum flowering/beginning of fruiting. Geographical occurrence data and the three months of maximum flowering time/beginning of fruiting were obtained from the herbarium specimen labels. The precipitation database with 30 s resolution from WorldClim – Global Climate Data (Hijmans et al., 2005) was imported in DIVA GIS version 7.5 (Hijmans et al., 2001), which was then used to determine the two precipitation values for each taxon occurrence points. Annual average and flowering/beginning of fruiting precipitations were then treated as continuous characters in the genus phylogeny and analyzed using the parsimony reconstruction and correlation methods of the PDAP package (Garland et al., 2002) implemented by Mesquite.

Structure and ultrastructure of stomatiferous protuberances (SPs)

Structure may reflect a particular function. Since extrafloral nectaries (ENs) were examined in detail by Schaffner (1979), we studied only the structure and ultrastructure of stomatiferous protuberances (SPs). Stem and flower samples of *C. gronovii*, *C. costaricensis*, *C. cotijana*, and *C. bonafortunae* were fixed directly in the field between 11 am and 12 pm using 3% glutaraldehyde + 2%