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BRIEF COMMUNICATION

Anatomical and histochemical characterization of *in vitro* haustorium from roots of *Castilleja tenuiflora*

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Abstract

In vitro induction of haustoria from *Castilleja tenuiflora* Benth. was achieved by applying 25 μM catechin, 25 μM vanillin, or 25 μM H_2O_2. Of the treatments tested, 25 μM vanillin was the strongest inducer of haustoria in *C. tenuiflora* roots *in vitro* (up to 3 haustoria per root). Haustorium development occurred laterally and was observable 14 d after inducer application. It was characterized by elongation of the epidermal cells and division of the inner cortical cells which also possessed abundant nuclei. Histochemical analysis using 3,3-diaminobenzidine (DAB) and diphenylboric acid 2-aminoethyl ester (DBPA) indicated that the formation of haustoria was associated with the accumulation of H_2O_2 and flavonoids.

Additional key words: catechin, flavonoids, hemiparasitism, hydrogen peroxide, vanillin.

Hemiparasitic plants rely partially on one or more neighboring plants (host) for acquisition of water, sugars, minerals, and secondary metabolites and for physical support. They have the ability to penetrate neighboring plant tissues to acquire water and solutes through an invasive structure called the haustorium which links the vascular systems of the two plants (Yoder 2001). Haustoria may form at the radicle or root apex and at lateral positions on the mature root in response to chemical signals released by host roots into the rhizosphere (Hood *et al.* 1998, Jamison *et al.* 2001). Signals include various compounds, such as quinones, hydroquinones, phenolic acids, flavonoids, and strigolactones (Albrecht *et al.* 1999, Cardoso *et al.* 2011). Haustoria induction involves molecular and biochemical events that are not fully understood. For instance, cell wall peroxidases requiring H_2O_2 as a co-substrate catalyze the oxidation of host cell-surface phenols into benzoquinones which are the signals mediating the development of haustoria (Keyes *et al.* 2001). Pectinolytic enzymes secreted by the parasite, such as polygalacturonase or rhamnogalacturonase are involved in the invasion process (Veronesi *et al.* 2007). Benzoquinones regulate the expression of the expansin genes *saExp1* and *saExp2* implicated in cell enlargement, one of the key features of haustorium formation (O'Malley and Lynn 2000). Hydrogen peroxide released by the parasite is critical for host recognition (Albrecht *et al.* 1999, Keyes *et al.* 2007).

The development of root haustoria begins with the cessation of parasite root tip growth, followed by isodiametric expansion of cortical cells within the root that results in a noticeable bulge. Epidermal cells elongate to form haustorial hairs capable of adhering to host tissues (Dobbins and Kuijt 1973, Hood *et al.* 1998, Torres *et al.* 2005). Once the parasite is attached to the host, a series of physical and enzymatic processes occur until the parasite reaches the host stele and establishes a

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Abbreviations: DAB - 3,3-diaminobenzidine; DBPA - diphenylboric acid 2-aminoethyl ester; DMBQ - 2,6-dimethoxy-*p*-benzoquinone; EC - epidermal cells; ICC - inner cortical cells; MZ - meristematic zone; SG - starch grain; NZ - zone with nucleus.
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connection between the host and parasite vascular systems (O'Malley and Lynn 2000). Hemiparasites have been studied rarely despite their important roles in ecology and agriculture (Deeks *et al.* 1999). Haustorium development can be monitored *in vitro* by applying host exudates or chemical compounds to aseptic plant cultures (Tomilov *et al.* 2005). Factors, such as the origin of the exudate and the nature of the haustorium inducer or its concentration need to be established (Riopel and Musselmann 1979). In *Triphysaria versicolor*, 2,6-dimethoxy-p-benzoquinone (DMBQ) and the anthocyanin peonidin were the most active haustorium inducers, although the concentrations at which they were active varied widely (Albrecht *et al.* 1999).

The genus *Castilleja* (family *Orobanchaceae* formerly *Scrophulariaceae*) comprises more than 220 herbaceous annual or perennial species distributed throughout the Americas (Tank *et al.* 2009). Most *Castilleja* species are ornamental and a few have medicinal uses. *Castilleja* spp. are autotrophic hemiparasites capable of independent growth but also associate with their hosts *via* haustoria (Dobbins and Kuijt 1973). Haustoria from natural-growing *Castilleja lutescens* and *C. cusickii* have been described by Dobbins and Kuijt (1973). *Castilleja tenuiflora* is a Mexican medicinal herb whose traditional use is associated with the accumulation of iridoid glycosides (Jiménez *et al.* 1995, Trejo-Tapia *et al.* 2012), phenylethanoid glycosides (Gómez-Aguirre *et al.* 2012), and flavonoids (López-Laredo *et al.* 2012). Understanding the hemiparasitic nature of this species is an important step for its protection and cultivation. The aim of the present work was to induce *in vitro* haustoria in roots of *Castilleja tenuiflora* and to characterize them anatomically and histochemically.

Fourteen-day-old *Castilleja tenuiflora* Benth. plants were obtained from shoots derived from the axillary buds of wild-grown individuals as described previously (Martínez-Bonfil *et al.* 2011). For the experiments, plants were grown in 50-cm^3 test tubes (*Sigma-Aldrich*, St. Louis, MO, USA) with 10 cm^3 of Schenk and Hilderbrandt (1972; SH) culture medium supplemented with sucrose (30 g dm^{-3}), 3-indole acetic acid (IAA, 10 μM) and 0.2 % (m/v) *Phytagel* (*Sigma-Aldrich*). Roots were grown along the surface of the medium which was solidified at a near 45° angle (Fig. 1*A*). Haustorial inducers were diluted in distilled water and applied to the roots of 21-d-old *C. tenuiflora* plants. The tubes were then kept at a near 45° angle for 2 h. Under these conditions, each plant presented an average of 35 roots. The following compounds were tested as haustorium inducers: vanillin (at concentrations 0 - 50 μM), 25 μM catechin, and 25 μM H_2O_2. Plants were maintained in a growth room at temperature of 25 ± 2 °C, relative humidity of 98 ± 2 %, a 16-h photoperiod, and irradiance of 70 μmol m^{-2} s^{-1}. The percentage of plants forming haustoria and roots with haustoria, and the number of haustoria per root were recorded 21 d after the application of the chemicals. Observations and photographs were made using a dissecting microscope (model *SMZ 1500*, *Nikon*, Tokyo, Japan) equipped with a digital camera (model *DC330*, *Dage-MTI*, Tokyo, Japan).

The roots were fixed in formaldehyde:acetic acid:ethanol:water (FAA) 10:5:50:35 (v/v) and 0.8 g dm^{-3} sucrose for 24 h and then washed with 2 cm^3 of 0.2 M phosphate buffer (pH 7.2). The fixed samples were dehydrated in an ethanol-xylol series and gradually infiltrated with *Paraplast* (melting point 60 °C) using *Paraplast*:xylol (50:50, v/v) in an incubator at 60 °C for 24 h. The samples were placed in embedding cassettes (*Sigma-Aldrich*), filled with *Paraplast* and hardened at 4 °C. Longitudinal sections of 10 μm were obtained with a manual rotary microtome (model *RM2125RT*, *Leica Microsystems*, Heidelberg, Germany) using disposable steel blades. The sections were then stained with hematoxylin for cell nuclei (Bancroft and Stevens 2002) and crystal violet for meristematic zones (Lara *et al.* 2003). These sections were used in histochemical studies to detect flavonoids with diphenylboric acid 2-aminoethyl ester (DBPA) (Saslowsky and Winkel 2001).

H_2O_2 was detected using the 3,3-diaminobenzidine (DAB) staining method as described by Huang *et al.* (2011). Fresh root samples were immersed in a solution containing 1 mg dm^{-3} DAB dissolved in Tris buffer at pH 5. Samples were exposed to vacuum for 6 min and incubated in darkness at 4 °C for 24 h. The samples were then washed with the Tris buffer and mounted on slides. Observations and photography were carried out using a light microscope (model *Eclipse 80I*, *Nikon*) equipped with a digital camera.

In the present work, we report for the first time the induction of haustoria in *Castilleja tenuiflora* roots *in vitro* (Fig. 1*A*) using catechin, vanillin, and H_2O_2. Haustorium development occurred laterally following treatments of the cultures with the three tested compounds. This kind of haustorium has been described in other species of *Castilleja* growing in nature (Dobbins and Kuijt 1973) and termed 'secondary' since it does not involve the root tip. The non-treated roots were 1 - 2 mm thick, 0.42 cm long, presented hairs and formed lateral roots but did not present haustoria (Fig. 1*B*). The roots treated with the chemicals were thicker (4 - 5 mm) and longer (1.46 - 1.73 mm) than the control roots. Catechin (50 and 10 μM) was shown to enhance the length of primary and lateral roots in *Arabidopsis*, possibly through redistribution of auxins (Rani *et al.* 2011). In contrast, *Tritium aestivum* exhibits a decrease in root length after treatment with H_2O_2 (Lu *et al.* 2013). The percentage of plants forming haustoria was the same regardless of the chemical compound tested (Table 1). However, among these plants, more roots formed haustoria after treatment with vanillin (5.7 %) than with catechin (1.7 %) or H_2O_2 (2.1 %). Also, the number of haustoria per root was higher when the roots were treated with vanillin (1 - 3) compared with catechin (1) or H_2O_2 (1). Although H_2O_2 is clearly involved in signaling during the development of haustoria until now, it has not been tested as a haustorial inducer. Of the treatments tested, vanillin induced the

greatest response; therefore, it was tested in different concentrations (0, 10, 25, and 50 μM) (Table 2). The highest dose of vanillin (50 μM) had a negative effect on the percentage of the *C. tenuiflora* plants forming haustoria. The strongest *in vitro* haustorium induction in the roots of *C. tenuiflora* was observed at 25 μM vanillin. Vanillin (at 50 μM) has also been reported to induce haustoria in *Triphysaria versicolor* (Albrecht *et al*. 1999).

C. tenuiflora haustoria were observable 14 and 21 d after application of the haustorium inducers, in contrast to *Triphysaria versicolor*, where *in vitro* haustoria formation occurred 24 h after the application of 10 μM 2,6-dimethoxybenzonquinone (DMBQ) or 10 μM peonidin (Albrecht *et al.* 1999). In *Agalinis purpurea*, the first

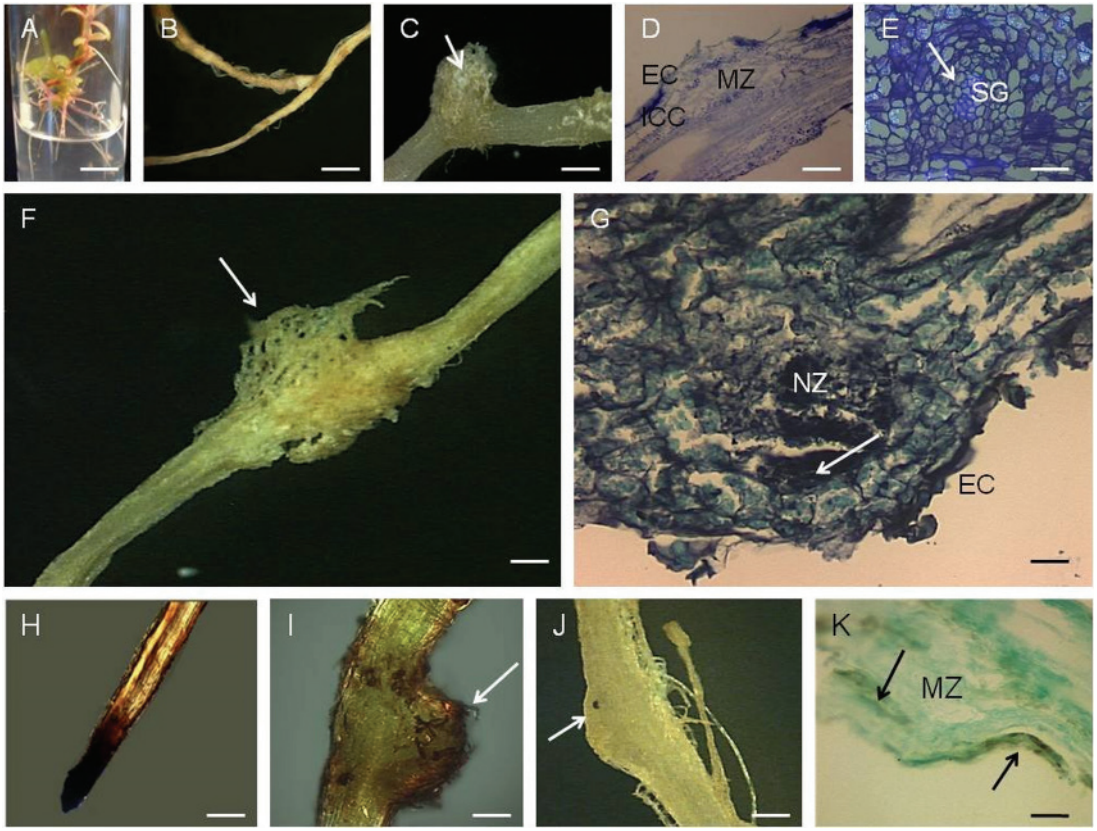

Fig. 1. Haustorium induction in *C. tenuiflora* roots with 25 μM vanillin. *A* - 21-d-old plants used for the experiments, *bar* = 5 mm; *B* - roots without a haustorium inducer treatment (control), *bar* = 1 mm; *C* - the secondary haustorium induced by vanillin, *bar* = 1 mm; *D* - the longitudinal section of a haustorium stained with crystal violet showing EC, MZ, and ICC, *bar* = 0.1 mm; *E* - the longitudinal section of a haustorium stained with crystal violet showing SG and meristematic cells with prominent nuclei, *bar* = 0.12 mm. *F* - the secondary haustorium induced by vanillin, *bar* = 0.1 mm; *G* - the longitudinal section of a haustorium stained with hematoxylin showing EC and MZ with prominent nuclei, *bar* = 0.1 mm; *H*, *I* - localization of H_2O_2 in roots using 3,3-diaminobenzidine; *H* - non-treated roots (14-d-old) showing reddish-brown staining at the tip, *bar* = 1 mm; *I* - the haustorium showing H_2O_2 accumulation at the surface and in the hairs, *bar* = 1 mm; *J*, *K* - localization of flavonoids using diphenylboric acid 2-aminoethyl ester; *J* - a root showing haustoria, *bar* = 1 mm; *K* - the longitudinal section showing yellow staining due to flavonoid accumulation, *bar* = 0.1 mm. *Arrows* indicate haustorium development, EC - epidermal cells, ICC - inner cortical cells, MZ - meristematic zone, SG - starch grains.

Table 1. Haustorium development in *C. tenuiflora* roots. Plants (21-d-old) were subjected to various haustorium inducers (at the concentration of 25 μM) and sampled 21 d after inducer application. Means ± SE from three replicated experiments (n = 30). Different letters indicate significant difference at $P < 0.05$ according to Tukey's test.

Compound	Plants forming haustoria [%]	Root length [mm]	Roots with haustoria [%]	Number of haustoria per root
Control	0	0.42 ± 0.1^{B}	0	0
Vanillin	75	1.73 ± 0.1^{A}	5.7 ± 1.0^{A}	1-3
Catechin	75	1.57 ± 0.1^{A}	1.7 ± 0.5^{C}	1
H_2O_2	75	1.46 ± 0.1^{A}	2.1 ± 0.5^{B}	1

Table 2. Haustorium development in *C. tenuiflora* roots after treatment with different concentrations of vanillin. Plants (21-d-old) were subjected to different concentrations of vanillin and sampled 21 d after inducer application. Means ± SE from three replicated experiments ($n = 30$). Different letters indicate significant difference at $P < 0.05$ according to Tukey's test.

Vanillin [μM]	Plants forming haustoria [%]	Root length [mm]	Roots with haustoria [%]	Number of haustoria per root
0 (control)	0	0.4 ± 0.1^{B}	0	0
10	75	2.1 ± 0.1^{A}	7.9 ± 2^{A}	1
25	75	2.2 ± 0.1^{A}	7.5 ± 2^{A}	1-2
50	25	2.5 ± 0.5^{A}	6.2 ± 1^{A}	1

cytological changes associated with the formation of haustoria occurs as soon as 30 min after the application of host exudates, but mature haustoria are only recognizable at 5 d (Riopel and Musselman 1979). Here, the haustoria induction response was low; up to 8 % of the *C. tenuiflora* roots formed at least one haustorium and each root presented up to three haustoria. This is in agreement with results reported for *Agalinis purpurea* (1.6 haustoria per root) (Baird and Riopel 1984) but it was lower than that reported for *Triphysaria versicolor*, where 73 % of the roots developed haustoria when exposed to 50 μM vanillin (Albrecht *et al.* 1999).

The *C. tenuiflora* roots (Fig. 1*A*) treated with vanillin were anatomically characterized and compared with the non-treated roots (Fig. 1*B*). After exposure to vanillin, lateral swellings of approx. 2 mm in length with hairs were visible along the *C. tenuiflora* roots (Fig. 1*C*). Another feature of haustorial formation was elongation of the epidermal cells (EC) and division of the inner cortical cells (ICC) which also showed abundant nuclei (Fig. 1*D*) similarly as observed by Baird and Riopel (1984) and Lee (2007). The radial enlargement and division of cortical cells is primarily responsible for initial swelling along the parasite root and gives rise to the haustorial primordium (Baird and Riopel 1984, Keyes *et al.* 2000). Starch grains (SG) were recognizable at the center of the surrounding meristematic zone (MZ) (Fig. 1*E*). These have been also observed in mature haustoria of *Castilleja* (Dobbins and Kuijt 1973) and may be used as an energy source for various cellular activities, including cell division and elongation during the development of the mature haustorium (Lee 2007). The cells constituting the center of these structures showed abundant nuclei (Fig. 1*F, G*) which is a characteristics of haustorial formation (Lee 2007).

Histochemical detection of H_2O_2 and flavonoids in *C. tenuiflora* haustoria was performed. The non-treated roots showed reddish-brown staining at the root tip (Fig. 1*H*) indicating accumulation of H_2O_2 in this zone. H_2O_2 was also found in the haustorial hairs and the epidermal cells of the haustoria (Fig. 1*I*). H_2O_2 has a role in rhizogenesis and other differentiation processes (Molassiotis *et al*. 2004, Kotis *et al*. 2009, Kärk and Koutaniemi 2010, Lu *et al*. 2013) as well as in constituting an essential part of the defense system (Keyes *et al.* 2001, 2007). H_2O_2 is also produced by parasitic plants at the site of host/parasite attachment (Keyes *et al.* 2007). Here, we show that H_2O_2 accumulates in certain zones of the *C. tenuiflora* haustoria which is in agreement with a report on *Striga asiatica*, where H_2O_2 accumulation is proximal to cells that developed into the haustoria (Keyes *et al.* 2007). During haustoria development, accumulation of secondary metabolites, such as flavonoids, may occur (Albrecht *et al.* 1999). In the present work, we detected flavonoids (yellow colour) at the epidermis and in the meristematic zone of the haustorium (Fig. 1*J*, *K*) suggesting that these structures might accumulate such secondary metabolites. These findings are under further investigation.

In conclusion, vanillin induced haustoria in the *C. tenuiflora* roots *in vitro.* The formation of these structures was associated with the accumulation of H_2O_2 and flavonoids.

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