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Endogenous hormone levels and anatomical characters of haustoria in *Santalum album* L. seedlings before and after attachment to the host

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ABSTRACT

The physiological and anatomical attributes of haustoria tissues in hemi-parasitic *Santalum album* L. seedlings, growing on the potential host, *Kuhnia rosmarnifolia* Vent., were investigated before and after attachment to the host. Quantization of endogenous levels of indole-3-acetic acid (IAA), zeatin (Z), zeatin riboside (ZR), GA-like substances (GAs) and abscisic acid (ABA) was performed by HPLC. Histological preparations were used to characterize structural differences between pre- and post-attachment haustoria. The contents of GAs and ABA were higher in attached haustoria, with 3.61 and 3.50 $\mu g\,g^{-1}$ fresh weight, respectively, and three times higher than in non-attached haustoria. Cytokinins, Z, ZR and IAA levels were also high, and their contents in attached haustoria increased 2.04-, 2.17-, and 2.82-fold more, respectively, than in non-attached haustoria. A high auxin-to-cytokinin ratio contributed to haustorial development of *S. album*. A numerous amount of starch in parenchyma cells around the meristematic region above the haustorial gland and the endophyte tissue of the post-attachment haustoria were reported in a Santalaceae member for the first time. Many lysosomes were present and large-scale digestion of host cells occurred at the interface between the parasite and host. The haustorial penetration in *S. album* into the host stele was suggested to be a function of mechanical force and enzymatic activity. Analysis of the endogenous hormone levels and the structural characters in *S. album* haustoria indicated that the haustoria were able to synthesize phytohormones, which appeared to be necessary for cell division and differentiation during haustorial development. These results suggest that endogenous hormones are involved in the haustorial development of *S. album* and in water and nutrient transport in the host–parasite association.

Introduction

The Santalaceae is a widely distributed family of flowering plants and its members, like other members of the Santalales, are partially parasitic on other plants. This means they obtain some of their water and simple nutrients by tapping into other plants even though they are able to manufacture their own complex compounds (Wanntorp and De Craene, 2009). The only economically important member of the family is *Santalum album* or sandalwood tree. It is a highly valued tree due to its aromatic heartwood, which contains sandal oil that is used in perfumes, cosmetics, medicine, and aromatherapy, and recently in the prevention of skin cancer (Kim et al., 2006a,b; Burdock and Carabin, 2008; Baldovini et al., 2011).

The tree is an obligate root hemi-parasitic tree. Cultivation of hemi-parasitic species is more complex than traditional monoculture (Radomiljac, 1998). In nature, as many as 300 species (including *S. album*) can act as hosts of sandalwood tree, supplying water and nutrients, through a unique organ termed the haustorium, especially during early phases of development (Radomiljac et al., 1998; Nagaveni and Vijayalakshmi, 2003). Parasitic angiosperms depend on host root-derived chemical signals to control various stages of development. For example, *Striga* species, which are obligate hemi-parasites, only germinate in response to host-borne root exudate components, the strigolactones (Kubo et al., 2009). Following this signal, most parasitic species will only develop a functional haustorium in the presence of a second chemical signal derived from the host, such as 2,6-dimethoxy-*p*-benzoquinone (DMBQ), phenolic acids, and flavonoids (Cook et al., 1966; Yoneyama et al., 2008). In the aseptic roots of the facultative parasite *Triphysaria versicolor*, haustorial development can be initiated by exposing the roots to phenolic derivatives exuded by the hosts' roots (Albrecht et al., 1999). Tomilov et al. (2005) identified the accumulation of auxin and ethylene as early events in

Abbreviations: ABA, abscisic acid; CK, cytokinin; GA, gibberellic acid-equivalent; IAA, indole-3-acetic acid; Z, zeatin; ZR, zeatin riboside.

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haustorium development of the hemiparasitic plant *T. versicolor*. *S. album* seeds pretreated with 2–8 mM gibberellic acid (GA_3) for 12 h could germinate *in vitro* on Murashige and Skoog medium (Nikam and Barmukh, 2009). *S. album* is an aggressive hemi-parasite with 70% of seedlings able to generate haustoria within 30 d from germination (Nagaveni and Srimathi, 1985). Barrett and Fox (1997) reported that haustoria were consistently found in all 3-month-old sandalwood seedlings grown in washed sand with a full nutrient regime. However, almost nothing is known about the factors inducing the formation of haustoria in *S. album*.

Plant hormones have long been known to play a crucial role in controlling plant growth and development. There exists to date some literature on endogenous hormone levels of the parasite/host association (Lechowski and Bialczyk, 1996; Frost et al., 1997; Jiang et al., 2004). Ihl et al. (1984) reported that the haustoria-bearing stem region in the *Cuscuta reflexa–Vicia faba* association had the highest levels of abscisic acid (ABA). The significant deposition of cytokinins (CKs), zeatin (Z), zeatin riboside (ZR), zeatin nucleotide (ZN), and ABA were detected in the haustoria of the *Rhinanthus–Hordeum vulgare* association (Jiang et al., 2004, 2005). These results suggest that the endogenous hormones in the haustoria of the parasite–host associations are likely to be somewhat related with its structure and function.

Previous work indicated that *S. album* is a xylem-feeding parasite relying on interfacial parenchyma to transport water and solutes (Tennakoon and Cameron, 2006). ABA can significantly increase plasma membrane permeability (Irving and Cameron, 2009) while phytohormones play a key role in vascular tissue regeneration (Aloni et al., 2006; Tokunaga et al., 2006). However, no information exists on endogenous hormone levels in the haustoria of the xylem-feeding parasite, *S. album*, before and after attachment to the host, although there are a few basic studies on the anatomy and development of *S. album* haustoria (Barber, 1906, 1907; Rao, 1942; Tennakoon and Cameron, 2006). However, other studies also indicated that the haustorium of the same parasite attached to the potential host plant showed differences in histological structure (Rao, 1942; Pate et al., 1990; Rumer et al., 2007). Therefore, additional studies are necessary to clarify the mechanism even further.

The purpose of this paper was to investigate the changes in cytokinins, Z and ZR, indole-3-acetic acid (IAA), gibberellic acid-equivalents (GAs; according to Agar et al., 2006), and ABA contents of *S. album* haustoria before and after attachment to the host plant, *Kuhnia rosmarnifolia* Vent., including those of the seedling root and the adjacent parasite and host root tissues, and to observe anatomical characters in the corresponding stages of haustorial development. Moreover, the links between endogenous hormone levels and the structural changes in *S. album* haustorium development were analyzed.

Materials and methods

Plant material

Fully ripe *Santalum album* seeds ($n = 400$) were obtained from a sandalwood tree introduced to the South China Botanical Garden. The endocarp was removed from the seed which were stored until required. The seeds were pretreated with a 1.44 mM GA_3 solution for 24 h at room temperature, and then sown in a sand bed at a depth of 2–3 cm in the greenhouse of the South China Botanical Garden. One month later, radicles 2–5 mm in length emerged and germinants were transferred to pots filled with a mixture of pond sludge, perlite, and peat (3:1:1, v/v/v) (20 cm in height, 20 cm in upper diameter, and 15 cm in lower diameter). About 20 d after planting, 300 actively growing and vigorous seedlings having two true leaves were obtained. The elite *S. album* seedlings were divided into three treatment groups, 100 per treatment (50 single *S. album* seedlings and 50 for the parasite–host association). *Kuhnia rosmarnifolia* is a potential pot host for *S. album* in China (Li, 2003). Stems of a 6-month old *K. rosmarnifolia* plant were cut into 10-cm-long segments with 3–4 lateral shoots and grown at a 15-cm row spacing in a sand bed at a depth of 2 cm in the greenhouse. The host seedlings, propagated by cuttings, and about 50 cm in height, were cut into 4–5 cm segments with two lateral shoots each. To obtain a *S. album–K. rosmarnifolia* association, the host segments were assembled 2–3 cm from the parasitic seedlings and 150 seedlings were inserted vertically into the soil, about 1.5–2 cm deep. The plants were cultivated in a greenhouse with a day/night temperature of 25 °C/18 °C and 70–80% relative humidity, with a 12-h photoperiod and light intensity of 180–260 mmol m^{-2} s^{-1}. During the experiment, the pots and sand bed were weeded when necessary and watered twice daily with tap water at 8:00 am and 18:00 pm.

The endogenous hormone levels of the haustoria, the parasite seedling roots and the adjacent parasite and host root tissues with haustoria were assessed separately. Seedling roots having two mature leaves were harvested, including 1–2 lateral roots (all root tips intact), corresponding to 20 d after planting. Pre-attachment haustoria about 1 mm in diameter were collected from roots when a single *S. album* seedling had six mature leaves about 45 d after planting. One hundred haustoria, each weighing about 1 g, were pooled. At 70 d after planting (5–15 d after attachment), and when seedlings had eight or ten leaves in the *S. album–K. rosmarnifolia* association, haustoria about 1.5–2 mm in diameter were isolated very carefully from the host root using a sharp cutter, and split at the connection between the haustorium and the parasite root tissue. The adjacent *S. album* and *K. rosmarnifolia* root tissues about 2 cm in length were harvested separately. The samples were quickly frozen in liquid nitrogen (N_2) and stored in −80 °C until use. Another sample was collected for anatomical observations.

Extraction, purification and determination of phytohormone

Extraction and purification of the cytokinins, Z and ZR, and IAA was conducted according to Agar et al. (2006) with a few modifications. The frozen samples (1 g) were powdered in liquid N_2, and 10 ml cold methanol containing 1 mM butylated hydroxytoluene as an antioxidant was added to the fine powder and stored at 4 °C for 24 h in the dark. The powder was filtered through Whatman No. 1 filter paper. The filtrates were pooled after the residue was re-extracted once more in the same way. The filtrates were filtered through 0.45 μm poly-tetrafluoroethylene (PTFE, Sartorius) filters. After evaporating off the methanol at 35 °C under reduced pressure with a rotary evaporator, the extract was redissolved in 0.1 M KH_2PO_4 buffer (pH 8.0) and centrifuged at 10,000 × *g* for 30 min at 4 °C. About 10 ml of the supernatant was transferred into a 25 cm^3 flask containing 1 g polyvinylpolypyrrolidone (PVPP, MW = 30,000, Sigma Chemical Co., UK), and then mixed well and filtered through Whatman No. 1 filter paper. The filtrates were loaded into Sep-Pak C18 cartridges (Waters, Hichrom Ltd., UK) after being activated with 2 ml of 100% methanol, followed by 2 ml of distilled water. Cartridge-absorbing hormones was loaded with 20 ml of ddH_2O and then eluted with 3 ml of cold 80% ethanol. After solvent evaporation, the dry residue was dissolved in 0.5 ml of 20% acetonitrile. The samples were filtered through a 0.45 Millipore filter and injected into HPLC to detect Z, ZR, and IAA.

The analysis of GAs and ABA were performed according to Kelen et al. (2004) and Agar et al. (2006), respectively with a minor modification. One gram of sample frozen was ground to a powder in liquid N_2 and homogenized in 3 ml of 100% methanol. The homogenate was stirred in 80% methanol at 4 °C overnight and then filtered through Whatman No. 1 filter paper. The residue was re-extracted with 80% methanol for 4 h, re-filtered and combined with