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Plant volatiles can minimize the growth suppression of epiphytic bacteria by the phytopathogenic fungus *Botrytis cinerea* in co-culture experiments

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Abstract

Leaf surfaces of plants are colonized by microbes, although the ecological roles of most of these epiphytes are unknown. Eleven non-pathogenic bacteria were isolated from strawberry (*Fragaria x ananassa*) plants and tested for their ability to interact with plant volatiles and the phytopathogenic fungus *Botrytis cinerea*. None of the bacterial epiphytes produced antimicrobial compounds. Light microscopic and SEM analysis of *F. x ananassa* leaf surfaces showed that capitate glands are densely colonized by microorganisms. Benzyl alcohol, 2-phenylethanol, *R*,*S*-linalool and nonanal were identified as major volatiles emitted by intact strawberry leaves, while *R*,*S*-linalool and nonanal were released by the capitate glands. The isolated epiphytes cannot utilize these leaf volatiles as sole carbon source, but some of the bacteria metabolize them, e.g. to the corresponding acids. However, the leaf volatiles have a stronger inhibitory effect on different strains of the plant pathogenic fungus *B. cinerea* than on the isolated epiphytic bacteria. In co-culture experiments, *B. cinerea* strains suppress the proliferation of epiphytes but low concentrations of 1–5 ppm of *R*,*S*-linalool, 2-phenylethanol and in particular nonanal significantly enhance the progeny of a number of epiphytic bacteria, while the growth of *B. cinerea* strains is retarded. Thus, native volatile compounds can affect population dynamics of epiphytes and the phytopathogenic fungus. Our findings have significant implications for pest management notably on the use of antagonistic bacteria as biocontrol agents.

Keywords: Epiphytic bacteria; *Botrytis cinerea*; Volatiles; *Fragaria x ananassa*

1. Introduction

Members of all plant phyla are colonized by microbial epiphytes, with more than 85 different species of microorganisms in 37 genera recovered from the phyllosphere of rye, olive, sugar beet, and wheat despite the hostile environment of the leaf surface (Morris et al., 1998; Yang et al., 2001). Leaf surfaces often are dry and strong UV radiation, limited nutrient availability, and large temperature swings contribute to stressful conditions. Epiphytes have been isolated only from a small number of commercially important crops but microorganisms as such from strawberry plant surfaces have not been investigated. However, the most common bacterial contaminants of strawberry explants, probably originating from epiphytes were recently characterized as *Pseudomonas fluorescens*, *P. corrugata*, *P. tolaasii*, *P. paucimobilis*, *X. campestris*, and *Enterobacter cloacae,* species also found on other plant surfaces (Tanprasert and Reed, 1998). It is assumed that the microbes present have a significant impact on stress resistance and on the plant's metabolism.

The interactions between plants and above ground epiphytic microorganisms have not been widely studied with regard to metabolite exchange. Pink-pigmented facultative methylotrophs (PPFM), however, are known to produce a variety of compounds, e.g. cytokinins, auxins, Vitamin B_{12},

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and osmoprotectants that affect plant metabolism and consume plant metabolic wastes, most notably methanol (Zabetakis, 1997; Joshi and Holland, 1999; Trotsenko et al., 2001). The composition and quantity of nutrients, including carbohydrates, organic acids, and amino acids that support the growth of epiphytic microorganisms, are affected by the plant species, leaf age, and the presence of tissue damage (Fiala et al., 1990; Hirano and Upper, 2000). Usually the carbon compounds or the nitrogen compounds are the limiting factors for bacterial and yeast populations on leaves (Wilson and Lindow, 1994; Ji and Wilson, 2002). The application of salicylic acid can alter the population size of salicylate-utilizing bacterial strains on leaves (Wilson and Lindow, 1995), suggesting that microbial populations of plants can be manipulated by changing the nutrient status of the plant surface. Thus, nutrient competition is a likely biological control mechanism for pathogens with a saprophytic growth phase or that require a nutrient source to infect plants (Brodie and Blakeman, 1976). However, to our knowledge, the effects of plant volatiles as potential nutrient sources or inhibitory compounds towards epiphytic microorganisms have not been thoroughly analyzed.

The antimicrobial properties of essential oils, odorous and volatile products of plant secondary metabolism are known for many centuries. A large number of essential oils and their constituents have been investigated for their antimicrobial properties against some bacteria and fungi in more than 500 reports. The majority of these reports deal with the susceptibility of human and food-borne as well as plant pathogenic bacteria and fungi towards different essential oils and their constituents (Croft et al., 1993; Kalemba and Kunicka, 2003). However, to the best of our knowledge, the effects of leaf volatiles on non-pathogenic epiphytes isolated from the same leaf species have not been tested.

Plant volatiles constitute a heterogeneous group of substances descending from the terpenoid, phenylpropanoid or fatty acid pathway. These lipophilic, low molecular weight compounds are emitted by flowers, fruits, stems, roots and leaves and are commonly found as constituents of plant resins and oils. Hundreds of volatile compounds have been identified as plant components, including substances, such as alcohols, aldehydes, ketones, esters, lactones, ethers, amines and carboxylic acids. A variety of specialized plant tissues as well as single cells secrete volatile compounds (Gershenzon et al., 1992; Hashidoko and Urashima, 1995). Extra-cellular secretion occurs mainly through different types of glandular trichomes, while internal secretion occurs through oil cells, cavities and ducts. Found in numerous plant families, glandular trichomes vary widely in shape and structure (Wagner, 1991). Two structural types of glandular trichomes are common. Peltate (subsessile) glandular trichomes, with a short, unicellular stalk and a large, multicellular head, accumulate secretory products in a cavity that forms between the cuticle and underlying cells, whereas capitate (clavate) glandular trichomes, with a one- or two-celled head atop a single- or multicellular stalk, excrete secretion products outside of the gland (Gershenzon et al., 1992). The exudates consist of a diverse group of alleochemicals and comprise monoterpenes (Rajaonarivony et al., 1992; Yamaura et al., 1992), sesquiterpenes (Hashidoko et al., 1992), phenylpropanoids (Gang et al., 2001) or other more unusual natural products, such as ω5-fatty acids (Yerger et al., 1992). Glandular trichomes that secrete terpenes and other essential oils have been found to confer insect resistance (Carter et al., 1989) but although terpenes and phenylpropanoids are known as antibacterial and antifungal agents (Moleyar and Narasimham, 1992), their effects on epiphytes have not been well studied.

The antagonistic properties of various epiphytes on phytopathogenic fungi, such as *Botrytis cinerea,* have been intensively investigated (Filonow et al., 1996; Guinebretiere et al., 2000; Berto et al., 2001). *B. cinerea* causes economic losses on a wide range of cultivated plants, stored fruits and vegetables. The pathogen is the major cause for the grey mould disease of grapevine and of the decay of strawberry fruit (*Fragaria x ananassa*). It is one of the most devastating pathogens in several crops worldwide (Paul et al., 1998). Although the effects of some leaf volatiles on pathogenic fungi have been described, their impact on epiphytes remains unclear (Hamilton-Kemp et al., 1992; Filonow et al., 1996; Manohar et al., 2001).

Owing to our experience with strawberry fruit flavor, and to the recent isolation and characterization of strawberry leaf epiphytes, the strawberry leaves are likely to provide a good model for assessing the role volatiles play in leaf surface microbial ecology. The goal of this research was to examine possible ecological functions of volatiles emitted by uninjured *F. x ananassa* leaves. We assumed that volatiles may serve as carbon source for epiphytes or inhibit phytopathogenic microorganisms, such as the fungus *B. cinerea*. However, the data suggest that volatiles might affect the population dynamics of epiphytic bacteria and the phytopathogenic fungus. This novel hypothesis has been investigated in detail using co-cultivation experiments. The obtained results have a significant impact on the use of bacterial biocontrol agents, since the co-application of volatiles and antagonistic bacteria could enhance the efficacy of the control agents.

2. Materials and methods

2.1. Strains

Epiphytic bacteria were provided by Prof. Lukas Schreiber, Institute of Cellular and Molecular Botany (IZMB), University Bonn, Kirschallee 1, 53115 Bonn, Germany and can be obtained there. Epiphytic bacteria were isolated and identified by morphological and biochemical methods as well as by their 16S rDNA (Table 1). The strains were maintained at −23 °C in Dworkin solution (2 g $(NH_4)_2SO_4$, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4$ in 1 l of distilled water) supplemented with 25% glycerol. A *Botrytis cinerea*