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Gall midge *Rhabdophaga rosaria*-induced rosette galls on *Salix*: morphology, photochemistry of photosynthesis and defense enzyme activity

Ineta Samsone, Una Andersone, Gederts Ievinsh*

Department of Plant Physiology, Faculty of Biology, University of Latvia, Kronvalda Bulv. 4, Riga LV-1586, Latvia

*Corresponding author, E-mail: gederts@lanet.lv

Abstract

Gall midge *Rhabdophaga rosaria* L. induces neoplastic formations on vegetative buds of *Salix*. Changes of chlorophyll content, chlorophyll a fluorescence, ascorbate peroxidase, polyphenol oxidase and peroxidase activity were analyzed in gall-leaves in comparison to adjacent control leaves in order to understand the nature of physiological changes caused by the gall-former. Chlorophyll content (R = 0.93), maximum photosynthetic efficiency of photosystem II (F_V/F_M; R = 0.83) as well as photosynthetic performance (R = 0.89) decreased in gall leaves from outer part towards the centre of the gall. Non-photochemical quenching increased in parallel with decrease in F_V/F_M towards the centre of the gall. Both peroxidase and polyphenol oxidase activity increased in gall leaves. In contrast, ascorbate peroxidase activity decreased with distance from the outer part of the gall. It is concluded, that the activity of the gall former affects both photosynthesis- and defense-related characteristics in galled leaf tissues. High activity of oxidative enzymes in a rosette gall tissues offers better protection of a gall-former against general herbivores and pathogens.

Key words: chlorophyll, chlorophyll *a* fluorescence, gall midge, galls, peroxidase, photochemistry of photosynthesis, polyphenol oxidase, *Rhabdophaga rosaria*, *Salix*.
Abbreviations: ETR, electron transport rate; F_V/F_M, potential maximum quantum yield or maximum quantum efficiency of photosystem II; ROS, reactive oxygen species.

Introduction

Plants respond to ovipositioning or feeding arthropods by inducing neoplastic growth at the site of disturbance. Neoplastic formations on trees cause long-term systemic effects on host tree phsyiology including biochemical characteristics with a possible adaptive role (Gailite et al. 2005).

Several biochemical components of adaptive value are of interest in the present context. Among them, polyphenol oxidase is a well known defense enzyme (Thypiapong et al. 2004) induced in response to herbivory (Constabel et al. 2000; Kruzmane et al. 2002). Another enzyme of defensive oxygen metabolism is peroxidase, induced by a wide variety of environmental stress factors, including wounding and herbivores (Forslund et al. 2000; Kruzmane et al. 2002). Among peroxidases, specific ascorbate peroxidase is a constituent of the enzymatic antioxidative system in chloroplasts and cytosol, induced as a response to unfavourable changes in many environmental factors (Kangasjärvi et al. 2008). On the other hand, changes in photosynthesis-related parameters during biotic interactions represent an important adaptive strategy when high metabolic costs of defense are considered. Recently it was shown that downregulation of photosynthesis-related genes is a general phenomenon during plant-herbivore interaction (Bilgin et al. 2010).

It has been shown that indirect plant defenses are manipulated by galling arthropods, leading to suppression of specific volatile synthesis in galled plants (Tooker, De Moraes 2008). It is more likely that direct defense responses (induction of oxidative enzyme activity, proteinase inhibitors etc.) are also suppressed in galled tissues. Thus, low activity of oxidative enzymes is a general characteristic of galls in numerous gall-former–host plant combinations, but this is not the case in *Rhabdophaga rosaria*-induced rosette galls on *Salix* (Gailite et al. 2005). On the other hand, generation of reactive oxygen species (ROS) in gall tissues could possibly lead to inhibition of photosynthesis through the direct and indirect effect on photochemistry of photosynthesis (de Oliveira et al. 2010).

Gall midges (Diptera: Cecidomyidae) represent the second largest group of gall-forming insects after cynipid gall wasps (Bográn et al. 2006). Several gall midge species of the genus *Rhabdophaga* form stem and shoot tip galls on willow (*Salix* L.) trees (DeClerck-Floate, Price 1994). In contrast to cynipid galls, where insect-induced modifications of plant development create novel plant

organs for protection and nourishing the developing larvae (Harper et al. 2004) rosette galls on *Salix* caused by a gall midge *Rhabdophaga rosaria* represent a result of development changes in existing vegetative buds. It can be expected that changes in biochemical characteristics of rosette galls might be less severe than in the case of "true" neoplastic formations e.a. cynipid galls.

The aim of the present investigation was to describe the morphology of rosette galls, changes of chlorophyll content, chlorophyll a fluorescence, ascorbate peroxidase, polyphenol oxidase and peroxidase activity in order to understand physiological and biochemical responses of possible adaptive value caused by the gall-former in *Salix-Rhabdophaga* galls.

Materials and methods

Gall formation was monitored from the middle of the June, which coincided with the development of new shoot elongation. Material for study was collected in July and August from adult *Salix* trees growing in the National Botanical Garden of Latvia, Salaspils, Latvia. *Rhabdophaga rosaria* galls were monitored on selected trees of *Salix alba* L., *Salix burjatica* Nasarov, *Salix caprea* L. and *Salix cinerea* L. Morphological measurements were performed on galled shoots of the selected trees.

Plant material for enzymatic analyses was weighed, frozen in liquid nitrogen and stored at –80 °C until use. Soluble protein extraction and measurement of polyphenol oxidase and peroxidase were performed as described previously (Gailite et al. 2005). Ascorbate peroxidase activity was measured according to Nakano and Asada (1987).

Chlorophyll content was measued in field conditions using a SPAD-502 chlorophyll meter (Konica-Minolta, Osaka, Japan). A near-linear relationship exists between SPAD values measured by a chlorophyll meter and spectrophotometrically determined total chlorophyll content on fresh mass basis (Samsone et al. 2007). Measurement was performed on individual leaves or gall-leaves with five consecutive readings across the surface of each leaf. The mean value was calculated by the internal function of the chlorophyll meter.

Chlorophyll *a* fluorescence was measured in field using two different methods: pulse amplitude modulated and continuous measurement. For pulse modulated measurement a portable fluorometer (PAM 2100, Walz, Germany) and leaf clip holder (2030-B, Walz, Germany) with integrated micro quantum-temperature sensor wasused. A laptop computer (Fujitsu Siemens Lifebook S7110) equipped with the appropriate software (DA-2000, Walz, Germany) was used to drive the measurements. Leaves were dark adapted for 30 min prior the measurements. First, potential maximum quantum yield (F_V/F_M) of photosystem II was measured. The minimal fluorescence level (F_0) was measured by low modulated light and the maximal fluorescence level (F_M) was determined by a saturating pulse on dark-adapted leaves. The ratio F_V/F_M was calculated, where F_V was the difference between the maximum fluorescence and the minimum fluorescence level F_0. After that, fluorescence induction curve with quenching analysis at 10 ms p^{-1} was recorded for 6 min using a built-in standard procedure of DA-2000. Maximum apparent electron transport rate through photosystem II (ETR) was calculated on the basis of measured overall photochemical quantum yield ($\Delta F/F_M'$; where $\Delta F = F_M' - F_S$) and of photosynthetically active radiation (PAR) according to the equation $ETR = \Delta F/F_M' \times PAR \times 0.5 \times 0.84$, assuming that transport of one electron requires absorption of two quanta (factor 0.5) and that 84% of the incident quanta is absorbed by the leaf (factor 0.84). Non-photochemical quenching (NPQ) was calculated according to the equation $NPQ = (F_M - F_M')/F_M'$.

For continuous measurement of chlorophyll *a* fluorescence, a Handy PEA (Hansatech, UK) fluorometer was used as descibed earlier (Andersone et al. 2010). The data were analyzed using the appropriate software (Hansatech, UK). Performance index was calculated, which represented the combination of the three independent parameters expressing accumulatively the respective responses of photosystem II: total number of active reaction centers per absorbtion, yield of primary photochemistry and efficiency with which a trapped exciton can move an electron into the electron transport chain (Appenroth et al. 2001).

Results

Morphology of galls

Galls on *Salix* caused by *Rhabdophaga rosaria* represent a case of neoplastic formations when vegetative buds are affected. Extremely fast development of rosette galls on *Salix* concomitant with growth of new shoots was evident. Growth of buds was initiated around June 15, while rosette gall initials (2 to 5 mm in diameter) were monitored on June 20. When sequential gall-leaves were collected and divided according to their shapes, several groups of leaves were clearly recognizable (Fig. 1). Characteristic changes of size and proportions in sequential gall-leaves led to formation of the particular shape of the gall, resembling a rose flower (Fig. 2).

As a result of the interaction between the gall midge and *Salix*, shoot elongation was completely suppressed, leading to formation of a swelled gall base (in average, 5.3 ± 0.3 × 4.8 ± 0.2 mm) to which gall-leaves were attached (Fig. 3B). The length of appropriate control shoots was 173 ± 18 mm for *Salix cinerea* and 206 ± 17 mm for *Salix alba*. The size of the gall base was positively correlated with the average size of the rosette gall. Several types of rosette galls were found on individual *Salix* trees, two of which (type A and B) had clearly distuinguishable morphological features. Galls of

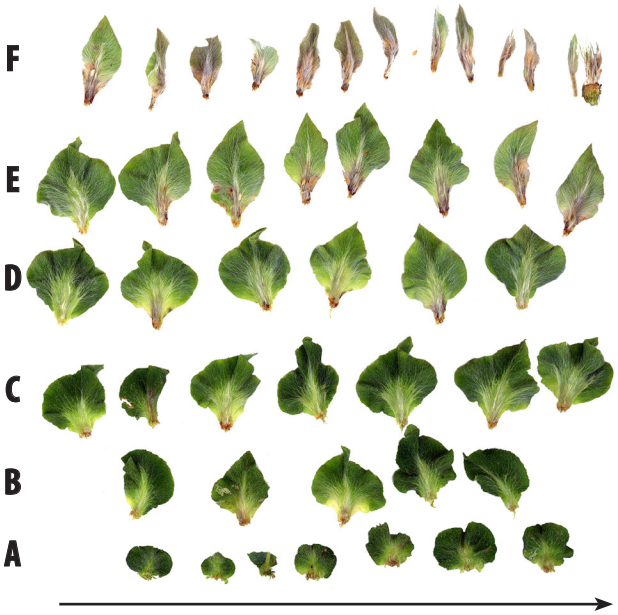

Fig. 1. Structure of a typical *Salix-Rhabdophaga* gall (type A) from the outer leaf (lower left) to the inner leaf (higher right). A – F, respective samples for chlorophyll, chlorophyll fluorescence and enzyme analysis.

the type A were relatively large, formed by clearly lanceolate to deltoid gall-leaves (Fig. 2A) with a gall base size of 6.2 ± 0.4 × 5.5 ± 0.3 mm. Gall-leaves of the smaller type B galls were more obovate, with central leaves lanceolate (Fig. 2B) with the gall base of 4.5 ± 0.2 × 4.2. ± 0.1 mm. This type was frequently found on individuals of *Salix alba* and *Salix caprea*. Type B galls tended to have lower number of gall-leaves per gall (37.1 ± 0.6) in comparison to type A galls (53. 3 ± 1.8).

Another morphological effect of gall-former activity was a change in number of leaves in gall structures vs. non-galled shoots. For *Salix alba*, the number of leaves on respective control shoots was 13.5 ± 0.8 (n = 15) while the number of gall-leaves per gall was 32.0 ± 3.1 (n = 12). In comparison, for trees of *Salix cinerea* the respective numbers were 12.6 ± 1.0 and 49.0 ± 3.7. An important characteristic of gall-leaves was complete reduction of petioles.

Gall-leaves were highly pubescent, with trichome density and length increasing towards the center of the gall (Fig. 3A). Orange-colored midge larvae and/or eggs were located between gall-leaves of the group F near the center

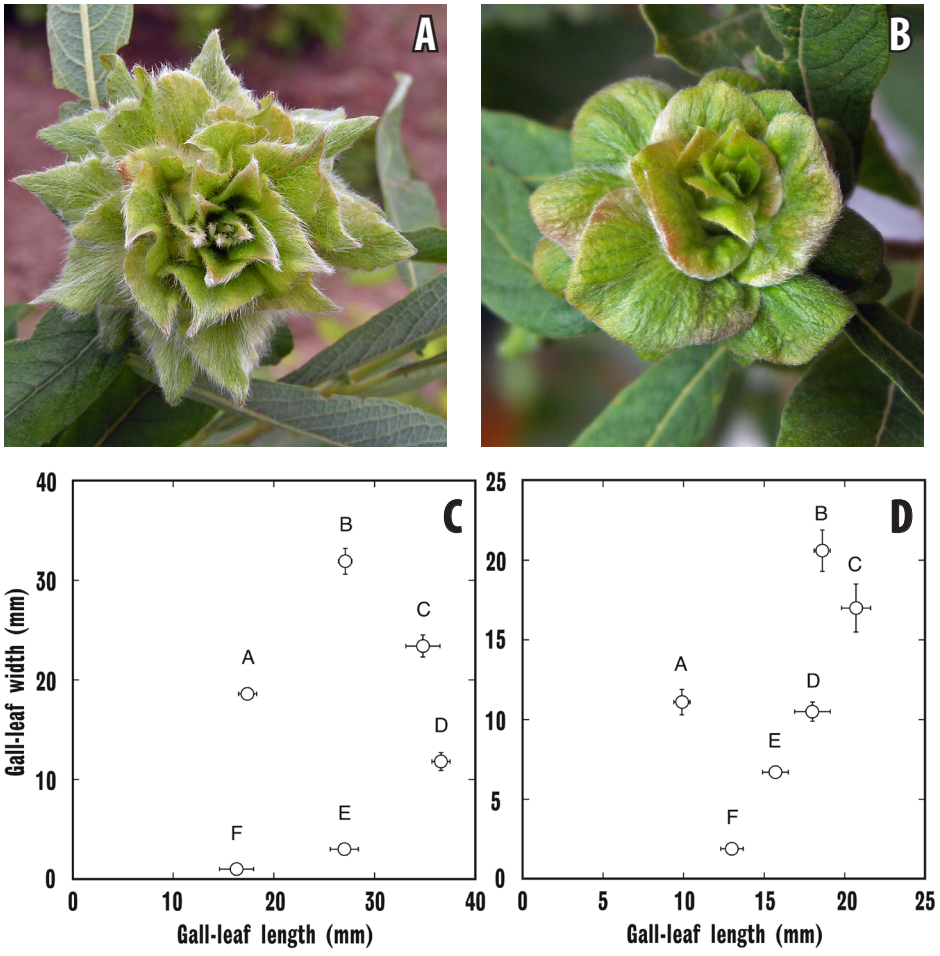

Fig. 2. *Salix-Rhabdophaga* galls of type A (A) and B (B) and the average size of gall-leaves ± SE in the respective groups of galls of type A (C) and B (D).

Fig. 3. Increase of trichome density on gall-leaves of a *Salix-Rhabdophaga* gall (A) and localization of midge larvae inside the gall structure (B). Bar = 1 mm.

of the gall (Fig. 3B).

Strong wound responses were seen in gall structures, indicated by an increase of brown coloring due to the accumulation of putative oxidized phenolic substances (Fig. 4). Coloring was localized in the basal part of gall-leaves and in the swelled part of reduced shoots. Consequently, increase of oxidative responses can be considered in galled *Salix* leaves.

Performance of photosynthesis and defense responses

When chlorophyll content and fluorescence characteristics were measured in different leaf groups from the outside towards the inside of the gall, there were no statistically significant differences in the parameters between the adjacent groups (Fig. 5). However, there was a tendency that both chlorophyll content and maximum photosynthetic efficiency (F_V/F_M), as well as electron transport rate (ETR) through photosystem II, decreased towards a center of the gall. A progressively increasing degree of photoinhibition of photosynthesis due to the direct damage of photosystem II was evident, as indicated by a gradual decrease of maximum efficiency of photosynthesis (Fig. 5B; F_V/F_M). In contrast, non-photochemical quenching increased toards the center (Fig. 5D). However, when sequential individual leaves of relatively small galls on *Salix caprea* were measured, chlorophyll content (R = 0.93), F_V/F_M (R = 0.83) as well as Performance Index (R = 0.89) linearly decreased in gall leaves from the outer part towards the center of the gall (Fig. 6). Both peroxidase and polyphenol oxidase activity increased in gall leaves (Fig. 7A, B). In contrast, ascorbate peroxidase activity decreased with distance from the outer part of the gall (Fig. 7C).

Discussion

Rhabdophaga rosaria-induced rosette galls on *Salix* trees of different species represent a unique case of neoplastic formation involving vegetative buds. Activity of the gall-former leads to complete inhibition of shoot elongation, induction of leaf formation with concomitant reduction of leaf growth and increased production of trichomes. No specialized nutritive tissues are formed, with gall-leaves

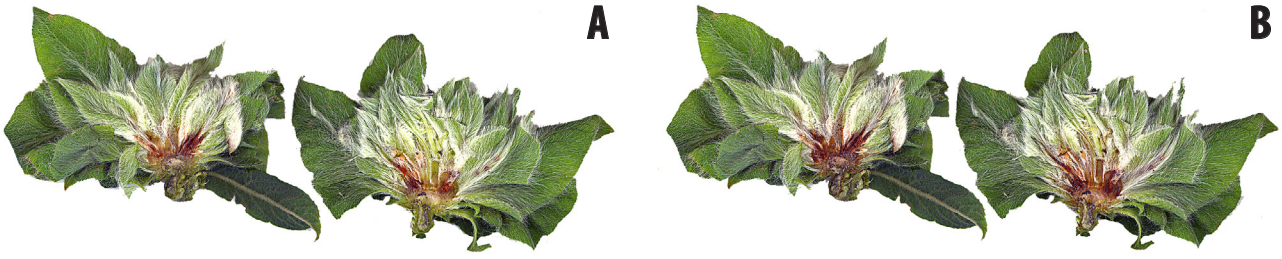

Fig. 4. Accumulation of oxidized phenolic substances in *Salix-Rhabdophaga* gall inner structures 30 sec (A) and 5 min after the wounding (B).

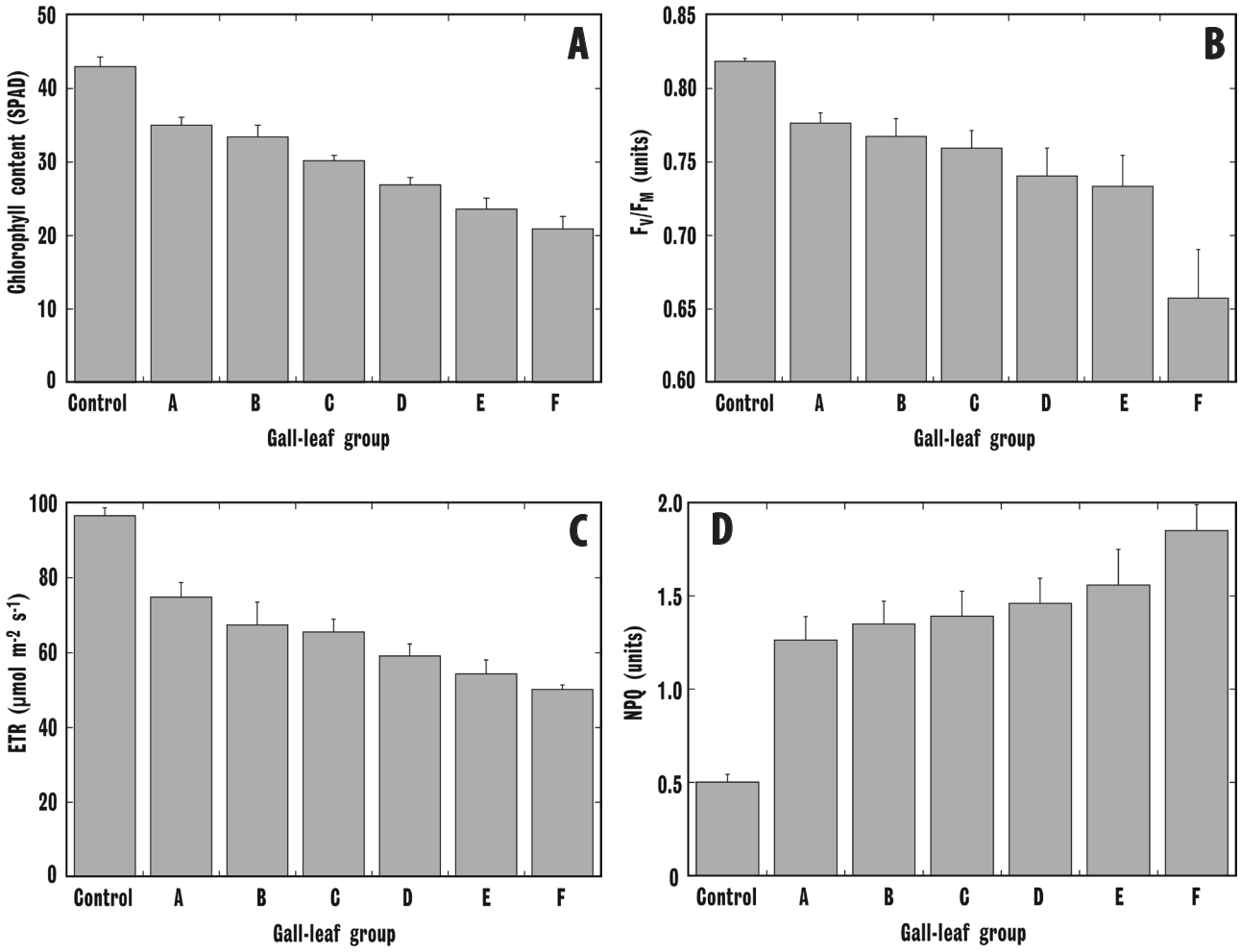

Fig. 5. Changes of chlorophyll content (A), maximum efficiency of photosynthesis F_V/F_M (B), relative electron transport rate of photosystem II (C), non-photochemical quenching (D) in different leaf groups of *Salix-Rhabdophaga* galls. As a control, leaves adjacent to gall on the same shoot were used. Data are means from at least 10 galls for every data point, five individual measurements per gall for chlorophyll content and three individual measurements for chlorophyll a fluorescence.

functioning both as a shelter and nutrient provider for developing larvae located in the center of the rosette gall. As larvae feed at the base of central gall-leaves, the biochemical characteristics of the leaves are of special importance for their development and protection.

Typical neoplastic tissues in general have suppressed defense enzyme activities (Gailite et al. 2005). In the present study, ascorbate peroxidase activity decreased towards the center of the gall while oxidative enzyme activties were higher in the central part of the gall. Downregulation of photosynthesis is another characteristic feature in biotic interactions characterizing a shift from "normal" physiology to a "defense" state (Bilgin et al. 2010). Neoplastic leaves of *Salix-Rabdophaga* galls clearly had fully functional photosynthetic apparatus, while vital characteristics of photosystem II photochemistry changed towards the center of the gall. Lower chlorophyll content, photoinhibition of photosynthesis, decrease in electron transport rate and increased thermal energy dissipation were among the most important of them, most evidently caused by larval feeding on central gall-leaves of the group F leaves (Fig. 1, 3B). The gradient of negative impact on chlorophyll content and photosynthetic performance diminishing from the rosette center outwards, possibly reflects direct wounding and/or a larval elicitor effect on host plant physiology. It has been argued that phloem-feeding arthropods constantly interact with plant metabolism at the sites of feeding (van de Ven et al. 2002). Similarly, the high activity of oxidative enzymes together with lowest activity of antioxidative enzyme ascorbate peroxidase in the central part of the gall reflect sub-systemic movement of exogenous or propagation of endogenous signals.

Reduced ascorbate peroxidase activity in gall-leaves indicates possible endogenous oxidative stress due to an insufficient capacity of the antioxidative system in comparison to the rate of ROS formation. Usually, there is an increase in ascorbate peroxidase activity in wounded plant tissues (Chang et al. 2004) or in tissues damaged by

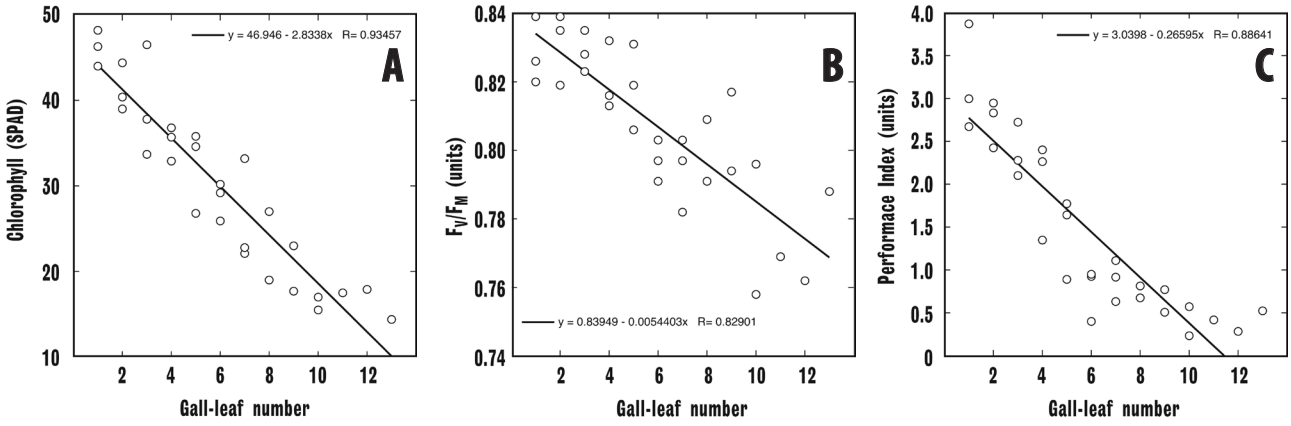

Fig. 6. Correlation between chlorophyll content (A), maximum efficiency of photosynthesis F_V/F_M (B), Performance Index (C) in individual leaves and leaf position in *Salix-Rhabdophaga* galls. The data represent individual measurements of all sequeuential gall-leaves from three individual galls. For chlorophyll content, five individual readings were performed and the mean value was calculated using an internal function of the chlorophyll meter.

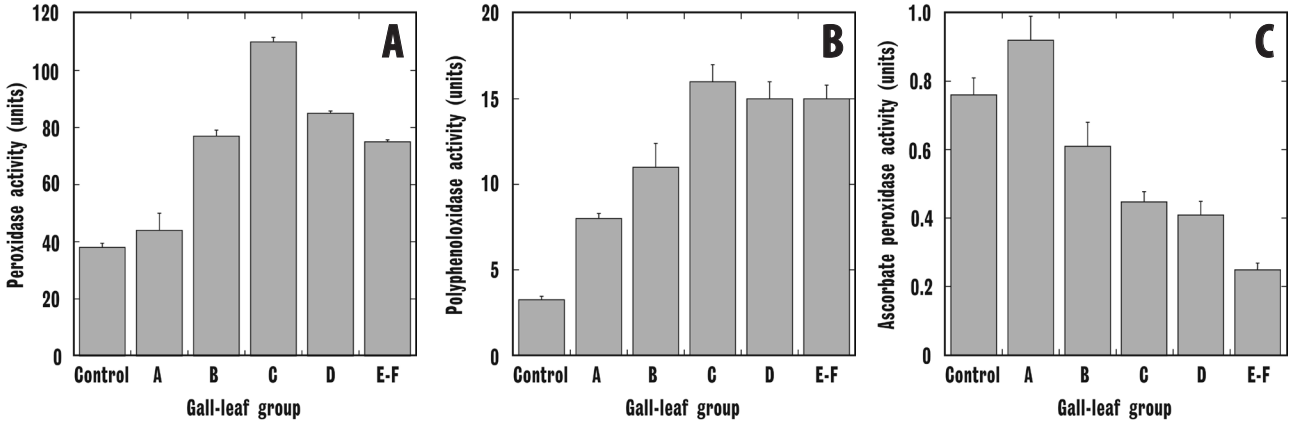

Fig. 7. Changes of peroxidase activity (A), polyphenol oxidase activity (B), and ascorbate peroxidase activity (C) in different leaf groups of *Salix-Rhabdophaga* galls. As a control, leaves adjacent to gall on the same shoot were used. Data are means from independent measurement of four samples for every data point ± SE. Each sample consisted of gall-leaves of the respective group from an individual gall.

chewing herbivores (Hu et al. 2009). In contrast, similar to the present data, phloem-sucking aphids cause a decrease in ascorbate peroxidase activity, while polyphenol oxidase and peroxidase activity are increased in the affected tissues (Khattab 2007).

In the context of decreased antioxidative protection, generation of ROS in gall tissues with possible negative consequences for photosynthesis has been proposed (de Oliveira et al. 2010). A significant decrease of maximum quantum yield of photosystem II (F_V/F_M) below 0.8, as shown in gall-leaves of *Rahbdophaga*-induced galls on *Salix*, is indicative of direct damage to components of photosystem II (Maxwell, Johnson 2000) resulting in photoinhibition of photosynthesis. Decreased chlorophyll content, relative ETR and Performance Index, as well as increased NPQ indicate reduction of overall efficiency of photosynthesis in gall-leaves.

Increased activity of oxidative enzymes is one of the antinutritive factors against arthropod herbivores (Zhu-Salzman et al. 2008; Barbehenn et al. 2010). Usually, insect performance is negatively related to high induced levels of oxidative enzyme activity. Thus, relatively high peroxidase activity, induced by a sawfly *Neodiprion sertifer* larvae feeding on *Pinus sylvestris*, led to decreased needle consumption and higher mortality of larvae in the next season (Andersone et al. 2009). Polyphenol oxidase produces phenolic oxidation products with a potential antinutritive activity through binding to essential amino acids (Felton et al. 1992). Numerous studies have related increased levels of plant polyphenol oxidase with negative effects on performance of caterpillars (Wang, Constabel 2004; Ruuhola, Yang 2006).

In the present study, both peroxidase and polyphenol oxidase activity increased in gall-leaves towards the center of the gall (Fig. 6), where midge larvae were localized (Fig. 2B). As a result, a high level of oxidative enzyme activity together with decreased protection against ROS formed a seemingly hostile internal environment for development of

gall midge. More likely, as in the case of highly specialized herbivores, developing larvae are protected against possible harmful consequences of a highly oxidative diet. Arthropod counter-defense could include the presence of multiple classes of midgut proteases ensuring effective digestion of defensive proteins of the plant origin (Brunelle et al. 1999), as insects have the ability to up-regulate detoxification systems in the case of induced plant defenses (Li et al. 2002). Similarly, increased polyphenol oxidase levels may have no negative effect on insect consumption or growth rates due to the low oxygen and high ascorbate content in insect gut (Barbehenn et al. 2007). On the other hand, high activity of oxidative enzymes in rosette gall tissues offers better protection against general herbivores and pathogens. As a result, more successful protection of the gall former might be ensured.

In conclusion, rosette galls of *Salix-Rhabdophaga rosaria* represent a specific case of defense state with changed morphology, lower defense against endogenous oxidative stress, suppressed photochemistry of photosynthesis through possible action of ROS, and increased activity of oxidative enzymes.

Acknowledgements

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