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RESEARCH ARTICLE

The OJIP fast fluorescence rise characterizes *Graptophyllum* species and their stress responses

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Abstract Causes for rarity in plants are poorly understood. *Graptophyllum reticulatum* is an endangered endemic species, and it has three close relatives with different conservation status: the vulnerable *G. ilicifolium*, the rare *G. excelsum*, and the common *G. spinigerum*. Applied to the chlorophyll *a* fluorescence transient of leaves, the JIP test provides a Performance Index (PI) which quantifies the main steps in photosystem II (PSII) photochemistry including light energy absorption, excitation energy trapping, and conversion of excitation energy into electron flow. The PI is calculated from three components which depend on the reaction center density, the trapping efficiency, and the electron transport efficiency. PI was measured in the natural habitats of the four species and under artificially imposed environmental stresses in the glasshouse to determine whether conservation status was related to stress resilience. The results showed that soil type is unlikely to restrict the endangered *G. reticulatum*, vulnerable *G. ilicifolium*, or rare *G. excelsum* because PI was similar in plants grown in diverse soils in the glasshouse. Photoinhibition is likely to restrict the endangered *G. reticulatum* to shade habitats because PI was significantly reduced when plants were exposed to more than 15% ambient light in controlled experiments. Water availability may determine the location and distribution of the vulnerable *G. ilicifolium* and common *G. spinigerum* because PI was reduced more than 60% when plants were exposed to water stress. While the characteristics of their natural habitats correspond to and explain the physiological responses, there was no obvious relationship between conservation status and environmental resilience. PI can be used to monitor vigor and health of populations of plants in the natural habitat. In cultivation experiments PI responds to key environmental variables that affect the distribution of species with conservation significance.

Keywords Fv/Fm ratio · JIP test · *Graptophyllum* · Performance Index · Photosynthesis · Rarity · Stress

Abbreviations

ABS	absorbance
C	common
Chl	chlorophyll
CS	cross section
DI	dissipation
E	endangered
ET	electron transport
Fm	maximum fluorescence level
Fo	minimal fluorescence level
Fv/Fm	maximum quantum yield of PSII photochemistry
PEA	Plant Efficiency Analyser
PI	performance index on absorption basis
PPFD	photosynthetic photon flux density
PQ	plastoquinone
PSII	photosystem II
Q_A	primary electron acceptor of PSII
R	rare
RC	reaction center
RWC	relative water content
TR	trapping
V	vulnerable

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Introduction

Causes of rarity in plants are commonly studied by comparing closely related congeneric species because this approach minimizes the confounding effects of disparate phylogeny (Gitzendanner and Soltis 2000; Kunin and Gaston 1997; Simon and Hay 2003). Rarity has often been addressed by comparing genetic diversity of rare and common vascular plant species, yielding equivocal results (Baskauf et al. 1994; Bradshaw 1987; Dodd and Helenurm 2002). It has been proposed that physiological studies of species in which intrinsic biology, biotic interactions and abiotic factors have failed to uncover the cause of rarity, may provide at least part of the answer for why some plant species naturally occur in small, restricted populations (Richards et al. 2003). Comparisons of ecological characteristics of restricted species and a widespread congener have already been considered as an approach for understanding causes for rarity, but not physiological characteristics (Baskauf and Eickmeier 1994; Walck et al. 2001; Witkowski and Lamont 1997). In a review by Bevill and Louda (1999) physiological attributes of rare and closely related more common plants were assessed, but no consistent differences were identified in the measured attributes of rare and common taxa. Hypotheses have been presented concerning causes and consequences of rarity that fall into three broad categories: history, genetics, and ecology (Baskauf and Eickmeier 1994; Walck et al. 2001).

In this study, we combined ecological and physiological approaches to explain differences in rarity of four rainforest *Graptophyllum* species in Australia. We hypothesized that different ecophysiological responses to abiotic conditions of restricted plant species compared with their widespread congeners are potential causes for rarity. Good indicators for plant adaptations to their environment are photosynthetic responses (Adams and Demmig-Adams 2004; Cavender-Bares and Bazzaz 2004; De Ronde et al. 2004; Loreto et al. 2004). We measured photosynthetic responses of the four subtropical shrub species with different conservation status for whom no significant difference in genetic diversity had been found (Shapcott 2007).

We tested the species' photosynthetic responses to soil transplants as well as artificially imposed high light and low water availability. By determining the species' resilience to common environmental limitations, we aimed to identify causes for rarity and possible threats for species survival due to environmental change.

Among the many experimental techniques available for investigation of the photosynthetic functions, chlorophyll (Chl) a fluorescence measurements have proven to be very useful (Adams and Demmig-Adams 2004; Baker and Horton 1987; Cavender-Bares and Bazzaz 2004). Chl fluorescence responds to environmental stress at the leaf level and is a rapid and non-destructive technique (Strasser et al. 2004). Chl fluorescence is a direct and integrated measure, making it a valuable tool for plant studies from leaf to ecosystem levels (Adams and Demmig-Adams 2004; Cavender-Bares and Bazzaz 2004; Strasser et al. 2000; Strasser and Strasser 1995). Recent improvements in detecting the complex fluorescence signal through direct, time-resolved measurements have provided detailed information on the fast fluorescence rise.

All oxygenic photosynthetic material investigated so far using this method shows the polyphasic rise with the basic steps from the 'origin' (O) through two 'inflections' (I_1, designated as J, and I_2, termed I) to a 'peak' fluorescence level (P) (Strasser et al. 2004). The analysis of the fast fluorescence rise according to the JIP test allows the derivation of several expressions leading to the actual description of a photosynthetic sample in a current physiological state. This technique has been used for screening the effects of light intensity, temperature, drought, atmospheric CO_2 or ozone elevation, and heavy metal contamination (Appenroth et al. 2001; Clark et al. 2000; Krüger et al. 1997; Moise and Moya 2004; Strasser et al. 2000, 2004; Strauss et al. 2006). The method was developed to obtain information about the fluxes of photons, excitons, electrons, and further metabolic events from one measurement (Strasser et al. 2004). Although the JIP test is an oversimplification of the energy flux theory, it nevertheless incorporates the in situ complexities of antenna structure such as pigment arrangement, exciton migration, and connectivity (Force et al. 2003).

Materials and methods

Natural populations of *Graptophyllum* species

Four native Australian species of the genus *Graptophyllum* (Acanthaceae) were studied. The species were ranked according to conservation categories as follows: endangered (*G. reticulatum*) > vulnerable (*G. ilicifolium*) > rare (*G. excelsum*) > common (*G. spinigerum*) (Briggs and Leigh 1996; Queensland Government 2000). All four species are understorey shrubs up to 4–5 m tall and frequently multi-stemmed. The conservation categories in which the four species in this genus are currently placed directly reflect the level of decrease in their geographic range and the number of populations. Only three populations of the endangered species *G. reticulatum* are known to exist in the Sunshine Coast region (Moreton district) and occur within 18 km of each other (Bean and Sharpe 1991; Shapcott 2007). The vulnerable *G. ilicifolium* has been found in three locations within an area of approximately 15 km diameter in the Mackay region, South Kennedy

district (ASGAP 2001; Barker 1986; Bean and Sharpe 1991; Nicholson and Nicholson 1995; Shapcott 2007). Less than 30 populations of the rare *G. excelsum* are known across its geographic range which comprises several hundred km from the districts of Cook, North Kennedy to Port Curtis, usually on soils derived from limestone (Barker 1986; Bean and Sharpe 1991; Queensland Herbarium 1993; Shapcott 2007). The common species (*G. spinigerum*) is sparsely but widely distributed, from Cape York and the Northern Territory in the far north of the country to near the Southeast Queensland border (Barker 1986; Shapcott 2007). According to Barker (1986), *G. spinigerum* is also found in Papua New Guinea.

Environmental characteristics

Climate data including rainfall, temperature as well as min/max temperatures were collected from weather stations (Bureau of Meteorology) nearest to the studied sites of the four species: Nambour (*G. reticulatum*), Mackay (*G. ilicifolium*), Rockhampton (*G. excelsum*), and Gympie (*G. spinigerum*). Ambient light intensity at the sites was determined using a light meter (Li-Cor, Inc., Lincoln, NE). Daily maximum temperatures and humidity at the sites were recorded using a digital hygro thermometer.

Soil samples were collected from the sites in the vicinity of the plants using a 10×10 cm^2 soil auger. The samples were collected from up to ten cores which were mixed to obtain a composite sample. Soil elemental nutrient content was determined after drying, sieving, extraction with triple-deionized water, and 1 M nitric acid (4:1 v:v) by inductively coupled plasma atomic emission spectrometry (ICPAES) (Rayment and Higginson 1992). The rate at which nitrogen and phosphorus are generated in the soil was determined with in situ ion exchange resin bags (Schmidt and Stewart 1998) over a four-day period at each sampling time. Three resin bags, each containing 5 g of resin in 50×50 mm^2 stapled polyethylene (Swiss Screens PE 48GG, 365 μm mesh), were inserted horizontally into the upper 5 cm of the soil. Sampling occurred in the peak of the wet season (February 2005). Resin bags were analyzed for nitrate (NO_3^-), ammonium (NH_4^+), and inorganic phosphate (Pi) concentrations calorimetrically (Schmidt and Stewart 1998). Soil samples were also analyzed for nitrate (NO_3^-) and ammonium (NH_4^+) following extraction with 2 M potassium chloride (Baethgen and Alley 1989; Rayment and Higginson 1992).

Glasshouse plants and experimental approach

Seedlings of all species were obtained from two native plant nurseries in southeast Queensland, and grown in pots in a naturally lit and ventilated glasshouse on the St Lucia campus of the University of Queensland. Plants were propagated by cuttings to produce sufficient plant material for the experiments and to minimize confounding effects of provenance. After 12 weeks of propagation in the glasshouse, uniform seedlings approximately 5 cm in size were transplanted into larger pots (25 cm depth, 10 cm diameter). Plants were acclimated to treatment conditions for 4 weeks before treatments were started. Pots were placed on wire trolleys ($1 \text{ m} \times 2 \text{ m}$ size) and the position of trolleys was changed weekly to minimize effects of climate gradients within the glasshouse. Four light intensities (12%, 20%, 40%, and 75% of sunlight) were generated using neutral shade cloth. Since transmission of light into the glasshouse was 75% of full sunlight, the highest light intensity chamber did not require shade cloth. Under 12% sunlight conditions, corresponding to approximately 140 and 230 μmol photons m^{-2} s^{-1} on a cloudless winter and summer day, respectively, all species had the highest Fv/Fm ratio, an indicator of plant health, at these light intensities. Therefore this light level was used for soil transplant and water stress treatments (see below).

Plants were divided into four groups: controls, soil transplant, excess light, and water stress treatments. Five individuals of each species were randomly assigned to each treatment. Experiments were carried out from March to November 2005, except for the water stress experiments which were conducted in December 2005.

Control plants were grown under 12% sunlight and in nutrient- and organic matter-rich potting mix (standard California potting mix). Before seedlings were transplanted, 30 g slow release fertilizer (Osmocote Exact 8/9 M, Scotts International BV, Heerlen, The Netherlands) were placed 10 cm below the pot surface. The fertilizer contained nitrogen (15%), phosphorus (4%), potassium (7.5%), and magnesium (1.8%), as well as trace elements. Plants were watered daily with deionized water to saturation with water draining from the bottom of the pots.

In the 'excess light treatment' plants were exposed to 75% of full sunlight, and potting media and watering regime were identical to the controls. Plants were acclimated for 4 weeks prior to commencement of measurements. All species produced new leaves 5 months after commencement of the 'excess light treatment' and these were measured as a separate leaf cohort.

In the 'soil transplant treatment' plants were transferred to soil collected from the natural habitat of the vulnerable *G. ilicifolium*, near Mackay. This soil had the lowest nutrient contents of all natural habitats (Table 1). The top 30 cm of soil was taken from the natural habitat, homogenized, and filled into pots. Through nutrient addition from fertilizer, control plants in the fertilized potting mix received up to 5 and 60 times more nitrogen and phosphorus, respectively, than plants in the 'soil transplant experiment.'

Table 1 Summary of formulae and definitions of some JIP test parameters

Parameter	Calculation	Description
Extracted and technical fluorescence parameters		
Relative variable fluorescence at 2 ms: V_J	= (F2 ms – Fo)/(Fm – Fo)	For unconnected PSII units, equals the fraction of closed RCs at 2 ms expressed as a proportion of the total number of RCs that can be closed.
Net rate of PSII closure: (dV/dt)o or Mo	= 4 (F300μs – Fo)/(Fm – Fo)	An approximation of the slope at the origin of the fluorescence rise (dF/dt)o which is a measure of the rate of the primary photochemistry. It is a net rate because the reduced Q_A can be reoxidized via electron transport beyond Q_A.
The flux ratios or yields		
Trapping probability or maximum quantum yield of primary photochemistry: ϕ_{Po} or TRo/ABS	= (1 – Fo)/Fm = Fv/Fm	The probability that an absorbed photon will be trapped by the PSII RC with the resultant reduction of Q_A. Relates to the whole measured sample that may be heterogeneous in terms of Q_A reducing and non-reducing RCs.
Electron transport probability: ψo or ETo/Tro	= 1 – Vj	The probability that an electron residing on Q_A will enter the electron transport chain.
PI	= $(\gamma_{RC}/(1-\gamma_{RC}))((\phi_{Po}/(1-\phi_{Po}))((\psi o/(1-\psi o))$ = $(RC/ABS)(P_{TR})(P_{ET})$	Multi-parametric expression of these three independent steps contributing to photosynthesis.
RC/ABS = $\gamma_{RC}/(1-\gamma_{RC})$	= $(Vj\cdot\phi_{Po})/Mo$ = [(F2 ms – Fo)/ 4(F300μs – Fo)]·(Fv/Fm)	The contribution to the PI of the active RC density on a Chl basis.
Performance due to trapping probability ϕ_{Po} (P_{TR}) [$\phi_{Po}/(1-\phi_{Po})$]	= Fv/Fo	The contribution to the PI of the light reactions for primary photochemistry
Performance due to electron transport probability ψo (P_{ET}) [$\psi o/(1-\psi o)$]	= (Fm – F2 ms)/(F2 ms – Fo)	The contribution to the PI of the dark reactions

After 32 weeks of growth under control conditions in the glasshouse (April–December 2005), the 'water stress treatment' was initiated. All plants were mature, as indicated by flowering, and 50–85 cm tall. Five plants per species were assigned to each control and water stress treatments. Controls were continued to be watered daily, while water supply was limited for the water stress plants. The treatment was continued until photosynthetic rates in mature leaves reached zero, which occurred after 20 days. Due to the limited volume of the pots, it was necessary to add some water to keep the water content of the potting media at approximately 20%. The water content (in %) of the substrate was estimated by the equation $(pot - pot_{dried})/(pot_{watered} - pot_{dried})$, where $pot_{watered}$ and pot_{dried} were the weights of pots with well-watered soil and with soil dried to constant weight, respectively. After 5 days, every pot of the water stress treatment was weighed and water was added, on average 50–70 ml every 3 days. Without such continuous compensation for evapotranspiration loss, irreversible damage (necrosis of youngest leaves) occurred within a few days (data not shown).

Chl fluorescence measurements

Chl fluorescence transients were measured with the Plant Efficiency Analyzer (Handy PEA; Hansatech Ltd., King's Lynn, Northfolk, UK). In the Handy PEA fluorescence activating light is provided by an array of three high-intensity light-emitting diodes which are focused via lenses onto the leaf surface to provide even illumination. The diodes provide red light of a peak wavelength of 650 nm, which is readily absorbed by the chloroplasts. The fluorescence signal is received by the sensor head during recording and is digitized in the control unit using a fast Analogue/Digital converter. The fluorescence signal is digitized at different rates depending upon the different phases of the induction kinetic. For the first 300 μs fluorescence is sampled at 10 μs intervals. This provides excellent time resolution of minimal fluorescence intensity (Fo) and the initial rise kinetics. The time resolution of digitization is then switched to slower acquisition rates as the kinetics of the fluorescence signal slow. A 1-s measurement records 120 data points (Handy PEA Manual User's guide).

All measurements were performed on the upper surfaces of the youngest fully expanded leaves following a dark adaptation period of 10 min using the leaf clips provided by the manufacturer. The minimum dark adaptation period required for the four species was determined in preliminary experiments according to the procedures described in the Handy PEA Manual. Light intensity was 3,000 μmol photons m^{-2} s^{-1} to generate maximal fluores-

cence intensity (Fm) for all species, with the gain adjusted automatically to 0.6 to avoid over scaling errors.

In the field, measurements were carried out in summer (January and February) and winter (July), 2005, with ten replicates (five selected plants and two leaves per plant) for each species. In the glasshouse, plants were acclimated to treatment conditions for a period of 4 weeks. Subsequently, measurements were performed on three selected plants per treatment on a monthly basis for 6 months. Three measurements were performed on separate leaves on each plant. An exception was the water stress treatment, where Chl fluorescence was measured weekly during the period of treatment which commenced after 32 weeks growth in the glasshouse.

Analysis of the Chl fluorescence transient: the JIP test

The JIP test (Strasser and Strasser 1995; Strasser and Tsimilli-Michael 2001; Strasser et al. 2000, 2004) was used to analyze each Chl fluorescence transient. The shape of the OJIP transient has been found to be sensitive to stress such as excess light, temperature, drought, atmospheric CO_2, or ozone as well as chemical influences (Appenroth et al. 2001; Clark et al. 2000; Krüger et al. 1997; Moise and Moya 2004; Strasser et al. 2000, 2004; Strauss et al. 2006), and our study confirms these findings. Figure 1 displays examples of the fast fluorescence transient of the four *Graptophyllum* species in control and various stress conditions. The effects of these stresses on the transients of all four species were different, i.e., the change of the shape under stress conditions was different.

The following data from the original measurements were used: maximal fluorescence intensity (Fm), minimal fluorescence intensity (Fo), fluorescence intensity at 300 μs (F300 μs) required for calculation of the initial slope (Mo) of the variable (V) component of the transient, and the fluorescence intensity at 2 ms (the J-step) denoted as F_J.

The JIP test represents a translation of the original data to biophysical parameters that quantify the energy flow through PSII. The initial stage of photosynthetic activity of a reaction center (RC) complex is regulated by three functional steps, namely absorption of light energy (ABS), trapping of excitation energy (TR), and conversion of excitation energy to electron transport (ET). The parameters which all refer to time zero (onset of fluorescence induction) of the flux ratios or yields are the maximum quantum yield of primary photochemistry (ϕ_{Po} = TRo/ABS = Fv/Fm) and the efficiency (ψo = ETo/TRo) with which a trapped exciton can move an electron into the electron transport chain beyond Q_A^-.

Recently, the performance index on an absorption basis, PI, was introduced as a multi-parametric expression of these three independent steps contributing to photosynthesis. The PI was calculated as (for a review see Strasser et al. 2000, 2004)

$$PI = [\gamma_{RC}/(1-\gamma_{RC})][\varphi_{Po}/(1-\varphi_{Po})][\psi o/(1-\psi o)]$$

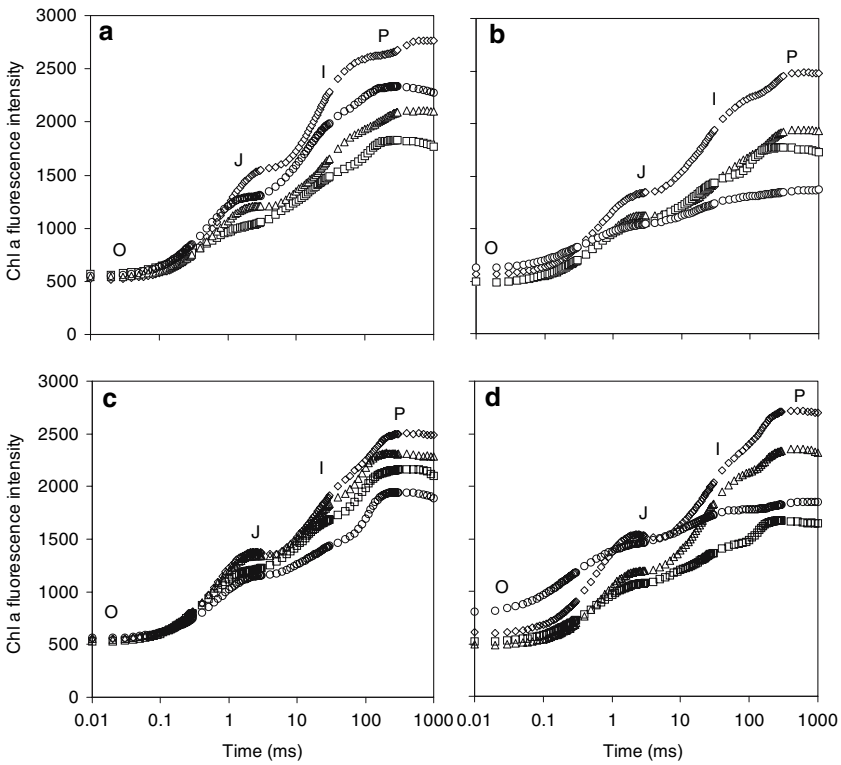

Fig. 1 The O-J-I-P fluorescence transient, plotted on a logarithmic time scale, of the four species: endangered *Graptophyllum reticulatum* (**a**), vulnerable *G. ilicifolium* (**b**), rare *G. excelsum* (**c**), common *G. spinigerum* (**d**) under control (◇), high light (□), poor soil nutrient (△) or water stress (○) conditions

where γ is the fraction of RC Chl (Chl_{RC}) per total Chl ($Chl_{RC+Antenna}$). Therefore, $\gamma/(1-\gamma) = Chl_{RC}/Chl_{Antenna}$ = RC/ABS. This expression can be de-convoluted into two JIP test parameters and estimated from the original fluorescence measurements as RC/ABS = [(F2 ms – Fo)/4(F300μs – Fo)]·(Fv/Fm). The factor 4 is used to express the initial fluorescence rise per 1 ms. The expression RC/ABS represents the active RC density on a Chl basis. The decrease of RC/ABS means an increase in the size of the Chl antenna serving each RC. The contribution of the light reactions to primary photochemistry is estimated according to the JIP test as $[\phi_{Po}/(1-\phi_{Po})]$ = Fv/Fo. This component of the PI represents the performance due to the trapping probability (P_{TR}). The contribution of the dark reactions is derived as $[\psi o/(1-\psi o)]$ = (Fm – F2 ms)/(F2 ms – F50 μs). It is the performance due to the conversion of excitation energy to electron transport (P_{ET}). The formulae in Table 1 illustrate how each of the above-mentioned biophysical parameters is calculated from the original fluorescence measurements (Strasser et al. 2000, 2004).

Among several parameters obtained from the Chl fluorescence measurements, the Fv/Fm ratio (= TRo/ABS) and the PI were selected for comparison of statistically significant differences. The reason for this choice was that the Fv/Fm ratio is the most widely used photosystem II (PSII) efficiency indicator. This parameter has been shown to correlate with the number of functional PSII complexes. Many studies have used this ratio as an indicator for stress tolerance or sensitivity (Cavender-Bares and Bazzaz 2004; Critchley 1998; Ogaya and Penuelas 2003). However, some studies have shown this parameter to be quite insensitive to change (Filella et al. 1998; Force 2002; Strasser et al. 2000; Strauss et al. 2006). Force et al. (2003) demonstrated the advantage of using a number of JIP test-derived fluorescence parameters to evaluate PSII function, rather than using only the Fv/Fm ratio. Recently (for a review see Strasser et al. 2000, 2004), the PI was introduced and has been used to quantify the effects of environmental factors such as chilling, heat, drought, chromate, ozone, or urban injuries on photosynthesis in several studies (Appenroth et al. 2001; Clark et al. 2000; De Ronde et al. 2004; Hermans et al. 2003; Strauss et al. 2006). According to the definitions of Strasser et al. (2000, 2004), the PI combines three values quantifying the three functional steps of photosynthetic activity by a PSII RC complex, from light energy absorption, trapping of excitation energy, and conversion of this energy to electron transport occurring in PSII. In the context of the O-J-I-P fluorescence transient, the PI is a function of the maximal and minimal fluorescence levels (Fm and Fo), the intermediate step J and the slope at the origin of the fluorescence rise. However, the Fv/Fm ratio is a function of only Fo and Fm and independent of the trajectory by which the fluorescence intensity reaches its maximal value. Research by Hermans et al. (2003) showed that PI is more sensitive to environmental change and correlates well with plant vigor and performance. In order to understand in more detail the response in structure and behavior of PSII to the environment, the three components of the PI were also compared.

Statistics

Statistical analyses were performed using SPSS 10.0 software (SPSS Inc.: Chicago, USA). One-way ANOVA (Bonferoni post-hoc test) at $P < 0.05$ was used to test whether there were significant differences within each species for each treatment and between the four species in the JIP test parameters.

Results and discussion

Environmental characteristics

Habitats of the four species differ in temperature, rainfall, soil properties including nutrient levels and pH, and light conditions (Table 2). Based on thermal regimes, Nix (1991) classified Australian rainforests into three groups: Megatherm, Mesotherm, and Microtherm. All four species grow in the understorey of subtropical rainforests, belonging to the Mesotherm group with annual mean temperatures centered around 18°C, ranging from 14°C to 22°C (Nix 1991). The endangered *G. reticulatum*, vulnerable *G. ilicifolium*, and common *G. spinigerum* grow in evergreen notophyll vine forest with higher annual rainfall (1,709, 1,665, and 1138 mm, respectively versus 946 mm of the rare species), but differentiation within these three species habitats depends on nutrients. Nix (1991) divided the evergreen notophyll vine forest group into three forest types, based mainly on differences in structure and soil nutrients. The endangered *G. reticulatum* occurs in complex notophyll vine forest with the highest soil nutrient, whereas the vulnerable *G. ilicifolium* and common *G. spinigerum* grow in notophyll vine forest with the lowest soil nutrients (Table 2). The habitat of the rare species (*G. excelsum*) is the semi-evergreen microphyll vine thicket, characterized with annual rainfall ranging from 551 mm to 1,483 mm. In partial compensation for drier conditions the soil nutrient status tends to be higher than habitats of the vulnerable *G. ilicifolium* and common *G. spinigerum*. It should be noted that the soil of the rare species (*G. excelsum*) habitat differs from the other soils as a consequence of being limestone derived and has higher pH (pH 7.0) and concentrations of plant available phosphorus and potassium (data not shown).

Table 2 *Graptophyllum* populations in the investigation of environmental variation

Species, site locations	Coordinates	Habitat	Annual rainfall, T mean	Soil	Ambient PAR (μmol quanta m^{-2} s^{-1})
G. reticulatum					
Site 1	Lat: 26°41′13″ Long: 153°04′18″	Complex notophyll vine forest	1,709 mm, 19.8°C	Basalt-derived Highest level of nutrient pH = 6.0	97
Site 2	Lat: 26°39′32″ Long: 152°54′20″				7
G. ilicifolium					
Mackay	Lat: 21°06′23″ Long: 148°54′10″	Notophyll vine forest	1,665 mm,22.5°C	Granite-derived Lowest level of nutrient pH = 4.9	25
G. excelsum					
Rockhampton Site 1	Lat: 23°09′14″ Long: 150°22′37″	Semi-evergreen microphyll vine thicket	946 mm, 23°C	Calcareous soil, Highest P content pH = 7.01	125
Site 2	Lat: 23°21′06″ Long: 150°34′24″				9
G. spinigerum					
Gympie	Lat: 26°13′45″ Long: 152°51′02″	Notophyll vine forest	1,138 mm, 20.3°C	Granite-derived Lowest level of nutrient pH = 4.23	20

Climate average data including rainfall, temperature were collected from the four weather stations (Bureau of Meteorology) closest to study sites of the four species. PAR data were presented as the values at midday, cloudless summer day

Ambient light intensities in the habitats of the four species, measured as photosynthetically active radiation (PAR) at noon on a cloudless day in summer, vary from 7 to 125 μmol photons m^{-2} s^{-1} which corresponds to 0.5–7.1% of full sunlight. The highest light intensity was recorded in the rare *G. excelsum* site (125 μmol photons m^{-2} s^{-1}), the lowest in the habitat of the endangered *G. reticulatum* (7 μmol photons m^{-2} s^{-1}). However, at one of the sites (foothills of Mt Etna) the rare *G. excelsum* was growing in very low intensity (9 μmol photons m^{-2} s^{-1}).

In summary, the endangered *G. reticulatum* grows in moist evergreen notophyll vine forest with high humidity and shading, and comparatively high soil nutrient levels. The rare *G. excelsum* grows in the driest sites in semi-evergreen vine thickets. Climate variables of vulnerable *G. ilicifolium* and common *G. spinigerum* differ from those of endangered *G. reticulatum* and rare *G. excelsum*, but soil nutrient levels in the habitat of vulnerable *G. ilicifolium* and common *G. spinigerum* were lowest.

Glasshouse experiment under control conditions

No significant difference was found in the Fv/Fm ratios of the four species when the plants were grown with adequate light, water, and soil nutrients in control conditions (Fig. 2a). PI values of the vulnerable *G. ilicifolium*, rare *G. excelsum*, and common *G. spinigerum* were similar and higher than this value of the endangered *G. reticulatum* ($P < 0.05$) (Fig. 2b). This result confirmed that the PI value is more sensitive than the Fv/Fm ratio, and that differences in PI exist between the species when grown under control conditions.

Figure 3 shows a comparison of the PI and its components between the three congeners and the endangered *G. reticulatum* under control conditions in the glasshouse.

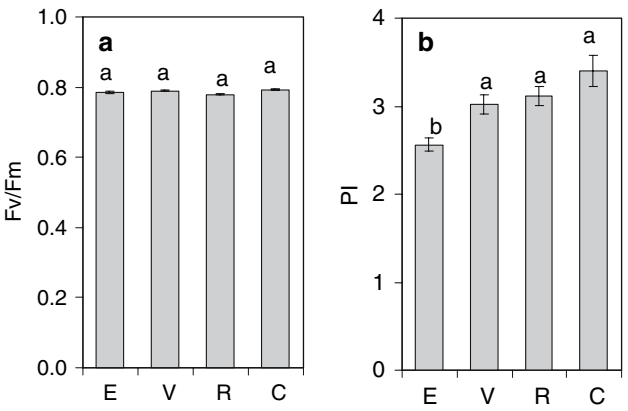

Fig. 2 Fv/Fm (**a**) and PI (**b**) of the four species, endangered *G. reticulatum* (E), vulnerable *G. ilicifolium* (V), rare *G. excelsum* (R), and common *G. spinigerum* (C), measured under control conditions in the glasshouse (12% sunlight, potting mix with fertilizer provided, watered daily to saturation). Significantly different means between the four species (ANOVA, Bonferoni post-hoc test, $P < 0.05$) are indicated with different letters. Values are averages of 30 replicates (±SEM)

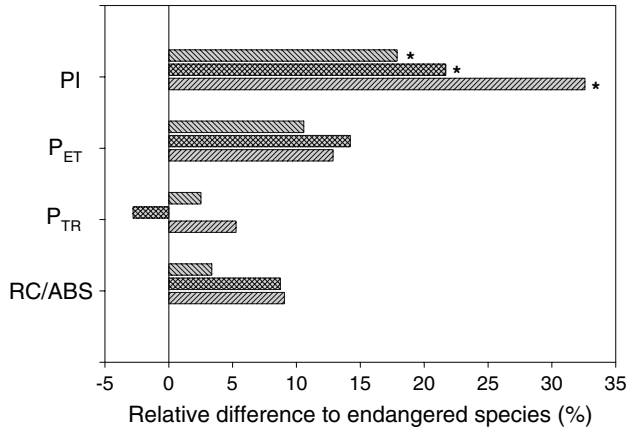

Fig. 3 Relative difference to the endangered *G. reticulatum* of the vulnerable *G. ilicifolium* (▨), rare *G. excelsum* (▩), and common *G. spinigerum* (▨) grown under control conditions in the glasshouse in the PI and its components: active RC density on a Chl basis (RC/ABS), performance due to trapping probability (P_{TR}), and performance due to electron transport probability (P_{ET}). The horizontal bars indicate the difference in the three more common plants relative to that of the endangered plants, calculated from the equation: Change to the endangered *G. reticulatum* (%) = (PI value of vulnerable, rare or common species – value of endangered species) × 100/PI value of the endangered species. The asterisk symbols indicate significant differences in PI and its components between the endangered and its relatives

The PIs of the three more widespread species were higher by between 15% and 35% with the dark reactions (P_{ET}) making the biggest contribution (11–14%) to these higher PI values.

Under optimum growth conditions the endangered *G. reticulatum* performed the least well, mostly because of a lower level of dark reaction activity. This is broadly consistent with the conservation status of the four species.

Soil transplant experiment

Fv/Fm values were very similar (no significant difference, data not shown) but PI was slightly reduced in all four species when grown in poor soil (Fig. 4a). This reduction was more severe in the endangered *G. reticulatum* and vulnerable *G. ilicifolium* (PI reduction >10%) than in the common *G. spinigerum* and rare *G. excelsum* (PI reduction <5%), with the greatest PI reduction of 13% observed in the vulnerable *G. ilicifolium*. The stronger decline in PI in the endangered *G. reticulatum* and vulnerable *G. ilicifolium* was accounted for by the decrease in trapping function (P_{TR}), suggesting an inactivation of RCs (Fig. 4a).

The soil transplant conditions differed from the control primarily by providing lower soil nutrient levels (see Methods section). When plants are nutrient-deficient, their growth rate is reduced (Evans 1996). According to Lambers et al. (1998), the photosynthetic machinery accounts for more than half of the nitrogen in a leaf, and

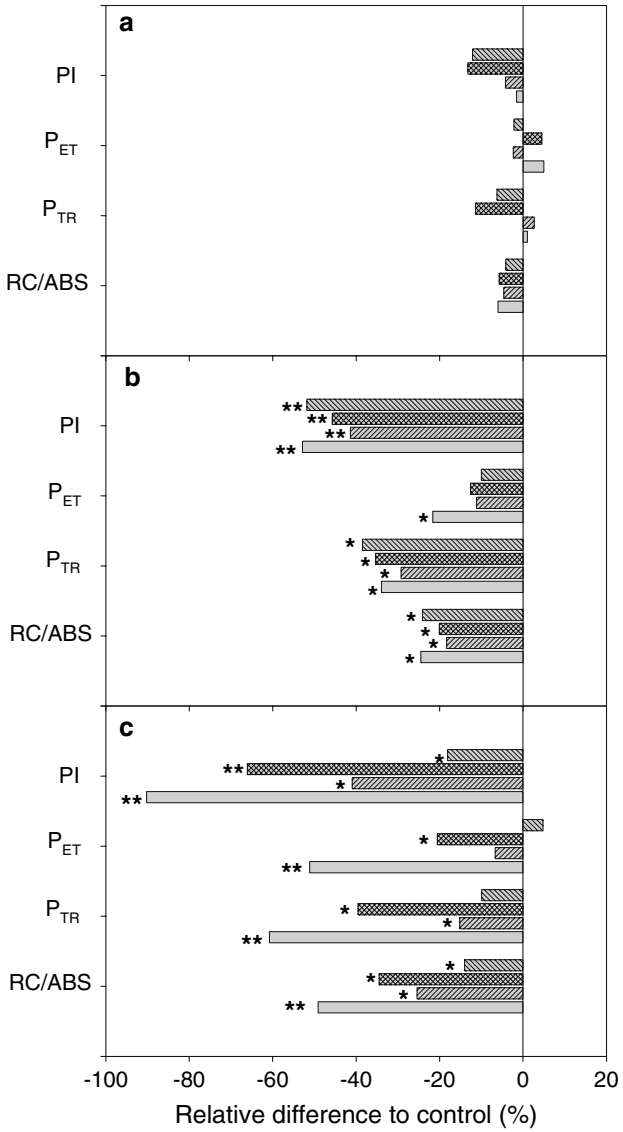

Fig. 4 The effect of soil transplant (**a**), 4 months excess light (**b**) and 17 days after imposing water stress (**c**) on the PI and its components of the four species: Endangered *G. reticulatum* (▨), vulnerable *G. ilicifolium* (▩), rare *G. excelsum* (▨), and common *G. spinigerum* (□). Values displayed as differences relative to control plants in the PI and its components: active RC density on a Chl basis (RC/ABS), performance due to trapping probability (P_{TR}), and performance due to electron transport probability (P_{ET}). The horizontal bars indicate the response in stress treated plants relative to that of control plants, calculated as (%) = ($value_{stress}$ – $value_{control}$) × 100/$value_{control}$. The asterisk symbols indicate significant differences in PI and its components for each species between the stress treated plants and control plants (ANOVA, Bonferoni post-hoc test, $*P < 0.05$, $**P < 0.001$)

photosynthesis is strongly affected by nitrogen availability. Contrary to this notion, however, the results obtained from our study showed that low nutrient supply did not have a major effect on Fv/Fm or PI in any of the four species. Similar results were obtained in a study on sunflower (*Helianthus annuus*). Ciompi et al. (1996) reported that

nitrogen deficiency in sunflower plants led to a reduction in CO_2 fixation rate, without a reduction in the maximum quantum yield of primary photochemistry, measured as the Fv/Fm ratio. According to these authors, the light reactions of photosynthesis of sunflower leaves were not influenced by nitrogen deficiency. The reduction in CO_2 assimilation at light saturation level was probably due to limitation in the functioning of the Calvin cycle as affected by reduced Rubisco activity, widely reported to occur under nitrogen deficiency (Chapin et al. 1988; Evans and Terashima 1987; Osaki et al. 1993). Further investigations are being conducted in our study using the gas exchange technique together with biomass determination to see whether this explanation also holds for the four species. However, to some extent the four species seem to be resistant to mild nutrient deficiency and the restricted distribution in their natural habitat must be due to factors other than soil nutrient content.

Excess light stress experiment

The Fv/Fm ratio fell significantly in all four species over the first 4 months and reached the lowest values after 3 or 4 months of transfer to high light conditions (Fig. 5a). A significant decrease in the Fv/Fm value constitutes photoinhibition and such changes indicate a loss of photochemical efficiency (Baker and Horton 1987; Critchley 1998). Over a 4 months period, the Fv/Fm values fell below 0.725 in all four species, a value considered to indicate photoinhibition (Critchley 1998). In terms of PI (Fig. 5b) the difference between the endangered *G. reticulatum* and its three more widespread relatives was even more pronounced: PI was reduced by 50% and did not recover within the experimental period. After 5 months, the three more widespread species had recovered from photoinhibition indicated by increases in Fv/Fm as well as PI. Only the endangered *G. reticulatum* remained photoinhibited, which demonstrated that this species was more vulnerable to high light.

Figure 4b shows the influence of 4 months excess light on the PI and its components. Under excess irradiance, all the components of the PI of all four species decreased. As a result, the PI dropped to 40–50% of that of the control plants. A decrease in P_{TR} (30–40%) contributed most to this reduction in PI, whereas decreases in RC/ABS and in dark reactions after Q_A^- (P_{ET}) contributed 20–30% and 10–20%, respectively.

The responses of the four species under high light conditions were consistent with previous studies of photoinhibition (Critchley 1998; Hikosaka et al. 2004; Lambers et al. 1998; Loreto et al. 2004). At high light levels leaves often absorb considerably more light than can

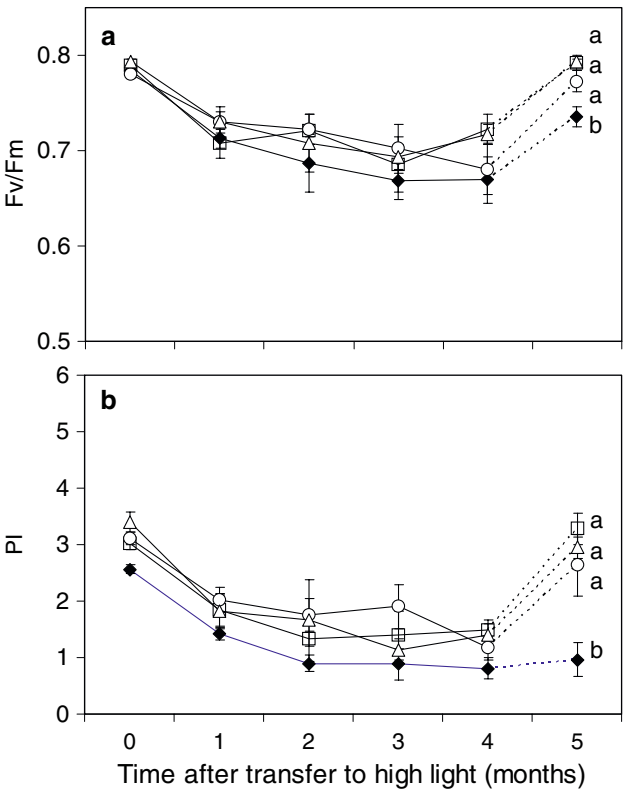

Fig. 5 Changes with time (months) after transfer to high light in Fv/Fm (**a**) and PI (**b**) of the four species: endangered *G. reticulatum* (◆), vulnerable *G. ilicifolium* (□), rare *G. excelsum* (○), or common *G. spinigerum* (△). Measurements were performed from May to October 2005 on leaves that existed at the time of transfer (1–4 months, *solid line*) and newly formed leaves (5 months, *broken line*). Different letters indicate significant difference between species after 5 months transfer to high light (ANOVA, Bonferoni post-hoc test, $P < 0.05$). Values are averages of nine replicates (±SEM)

be utilized for photosynthesis, creating a situation in which the photosynthetic apparatus could sustain photodamage with a consequent loss in photosynthetic productivity (Loreto et al. 2004). Plants have photoprotective mechanisms to dispose of this excess excitation energy such as thermal dissipation, the water–water cycle and cyclic electron flow around PSII to prevent the overreduction of quinone acceptors and photodamage to the PSII RCs (Hikosaka et al. 2004; Lambers et al. 1998). Therefore, dissipation can be thought of as the absorption of photons in excess of the trapping ability of the RC, indicated in this case by the rate of increase in the size of the Chl antennae serving each RC, i.e., decrease in RC/ABS. The decrease in P_{TR} mainly results from the inactivation of RCs, since absorbed energy could not fully be trapped by RCs, and would be dissipated through heat, fluorescence, and energy transfer to other systems. However, "switching off" some RCs occurs not only through damage, but also via a photoprotective acclimation strategy (De Ronde et al.

2004). The capacity of recovery from photoinhibition is an indicator of the capacity to recover RC function.

Figure 6 shows the effect of 5 months excess light on the PI, when leaves newly formed in high light were mature enough for measurements. The four species have different mechanisms to respond to prolonged high light. According to Adams and Demmig-Adams (2004) higher plants employ a multitude of approaches for dealing with excess excitation energy. There are a number of acclimatory and regulation adjustments that can be made within the chloroplast, such as alteration in light harvesting capacity, photosynthetic electron flow and excitation energy transfer efficiency (Adams and Demmig-Adams 2004). Under normal physiological conditions, the rate of photodamage does not exceed the capacity to repair the damage. When the excess light is prolonged, the PSII quinone acceptors do become highly reduced; the rate of damage can then exceed considerably the rate of repair (Baker et al. 2004).

PI in the endangered *G. reticulatum* continued to decrease (60%, $P < 0.001$). RC density per absorption (RC/ABS) decreased significantly (–40% compared to –25% after 4 months), indicating that long-term adjustments in the thylakoid membrane organization in leaves of this species driven by acclimation were not sufficient or incapable of responding quickly enough to changes in the growth light environment. Continuous decline in the performance due to trapping probability (P_{TR}) in this species

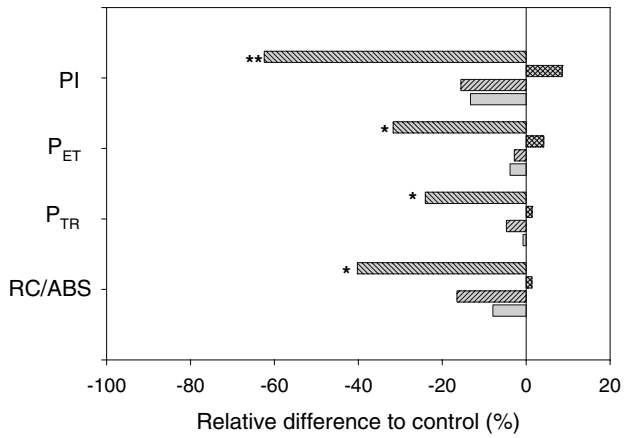

Fig. 6 The effect of 5 months excess light on the PI and its components of the four species: Endangered *G. reticulatum* (▨), vulnerable *G. ilicifolium* (▨), rare *G. excelsum* (▨), and common *G. spinigerum* (□). Values displayed as differences relative to control plants in the PI and its components: active RC density on a Chl basis (RC/ABS), performance due to trapping probability (P_{TR}) and performance due to electron transport probability (P_{ET}). The horizontal bars indicate the response in stress-treated plants relative to that of control plants, calculated as (%) = ($value_{stress}$ – $value_{control}$) × 100/$value_{control}$. The asterisk symbols indicate significant differences in PI and its components for each species between the stress-treated plants and control plants (ANOVA, Bonferoni post-hoc test, $*P < 0.05$, $**P < 0.001$)

indicated the inactivation of RC complexes resulting from the accumulation of damaged RCs. The contribution of the dark reactions, indicated by P_{ET}, in reduced PI value showed that excess light also was the cause for metabolic impairment. Therefore, we may conclude that the endangered *G. reticulatum* was more susceptible to high light and had a lower capacity for recovery from photoinhibition. This feature seems to be the reason for the restriction of the endangered species to shaded sites.

The recovery in the PI of the vulnerable *G. ilicifolium* to values above the controls (Fig. 6) showed that this species had a better mechanism for acclimation to high light. All three components of the PI increased significantly during this acclimation process. The P_{TR} was the greatest contribution, increasing from –35% to plus 1.4%, demonstrating a lesser degree of damage or greater capacity to replace RCs of PSII in this species. The greater increase in RC/ABS, observed in the vulnerable *G. ilicifolium* leaves compared with remaining species leaves, may indicate that leaves of the vulnerable *G. ilicifolium* were better able to regulate the amount of light reaching the RC and that this regulatory mechanism was perhaps not as effective in the other three species. According to Loreto et al. (2004), plants have evolved mechanisms to protect the photosynthetic apparatus from photodamage in all but the most severe situations. The vulnerable *G. ilicifolium* may reduce the amount of incident light that it absorbs through decreasing the number of light-harvesting Chl molecules. This was a significant change in the way the chloroplast allocated resources between harvesting and processing of absorbed light (Baker et al. 2004). The increase in the performance due to electron transport probability (P_{ET}) to a value higher than control plants (4%) of vulnerable *G. ilicifolium* clearly indicated some capacity for sun acclimation (Lambers et al. 1998; Lüttge 1997; Riddoch et al. 1991).

The rare *G. excelsum* also showed capacity for recovery from high light stress (Fig. 6). Similar to the vulnerable *G. ilicifolium*, the largest increase component was also the P_{TR}, demonstrating a capacity for repairing damaged RCs. The performance due to electron transport probability (P_{ET}) also improved from –11% to –2%. However, the RCs per absorption (RC/ABS) remained low, showing that this species did not have mechanisms of alteration in light-harvesting capacity, i.e., decreasing the number of light-harvesting Chl molecules to adapt to higher light conditions (Adams and Demmig-Adams 2004).

The common *G. spinigerum* responded similarly to the vulnerable *G. ilicifolium* plants to high light. Generally, all three components of the PI were increased after 5 months under high light leading to an increase in PI from –55% to approximately –15%.

Water stress experiment

The maximum quantum yield of primary photochemistry Fv/Fm fell slowly in all species over the first 11 days of imposing water stress (Fig. 7a). This slow decline continued in the endangered *G. reticulatum* and rare *G. excelsum*, so that Fv/Fm was still above 0.75 after 17 days when the CO_2 fixation rates had dropped to nearly zero (data not shown). In contrast, Fv/Fm of the common *G. spinigerum* declined at a dramatically increased rate and reached 0.6 after 17 days. The rate of decline in PI differed from Fv/Fm. The PI of the vulnerable *G. ilicifolium* and common *G. spinigerum* decreased quickly with a similar rate, whereas the endangered *G. reticulatum* and rare *G. excelsum* declined more slowly during the water stress period (Fig. 7b). Two groups can be classified after 17 days of imposing water stress, the endangered *G. reticulatum* and rare *G. excelsum* showed higher water stress tolerance, indicated by higher Fv/Fm ratio and PI value compared to the vulnerable *G. ilicifolium* and common *G. spinigerum* (Fig. 7a, b, $P < 0.05$).

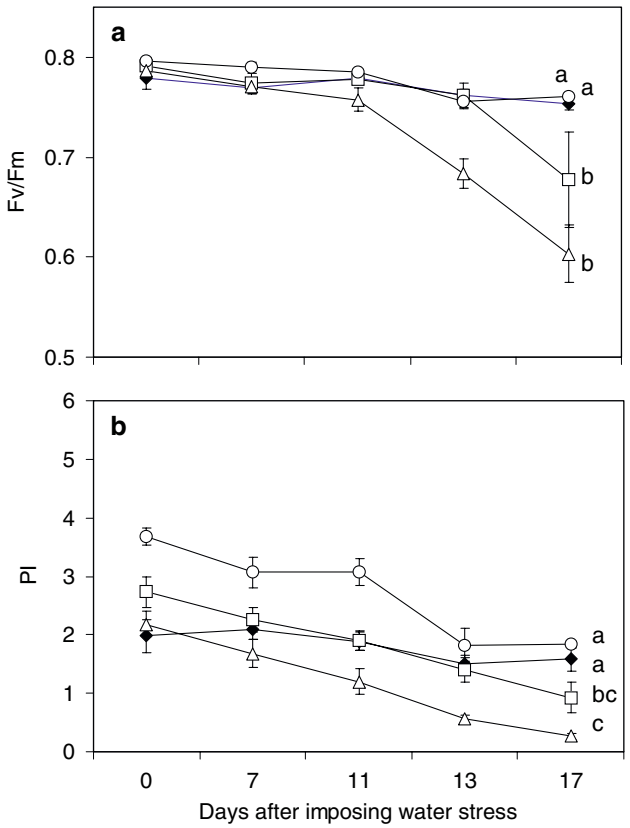

Fig. 7 Changes with time (days) after imposing water stress in Fv/Fm (**a**) and PI (**b**) of the four species: endangered *G. reticulatum* (◆), vulnerable *G. ilicifolium* (□), rare *G. exelsum* (○), or common *G. spinigerum* (△). Different letters indicate significant difference between species after 17 days imposing water stress (ANOVA, Bonferoni post-hoc test, $P < 0.05$). Values are averages of nine replicates (±SEM)

When plants are exposed to water deficits rapidly, leaf relative water content (RWC) decreases because water supply from the roots does not match water loss from leaves. According to Chaves et al. (2003), in this condition, thermal dissipation of absorbed light by non-radiative process plays a central role in leaf photoprotection. Leaves experience either a transient decrease of photochemical efficiency called 'down regulation of photochemistry" or they undergo photoinhibition. The slow decrease in Fv/Fm of the four species in the first 11 days of imposing water stress indicate the operation of 'down regulation' or photoprotection. The four species seem to be drought tolerant species because the photosynthetic apparatus in their leaves is resistant to lack of water and PSII functioning and its regulation are not qualitatively changed during mild water stress (Cornic and Fresneau 2002). However, when water stress was prolonged (after 11 days) photo-oxidative destruction of the photosynthetic apparatus in leaves of the four species might occur.

Figure 4c shows that 17 days imposing water stress had a negative impact on all PI components in all four species, the effect being least severe in the endangered *G. reticulatum*. It can also be seen from Fig. 4c that all three components of the PI in leaves of vulnerable *G. ilicifolium* and common *G. spinigerum* declined quite dramatically, varying from –60% to –20%, whereas these changes in leaves of the endangered *G. reticulatum* and rare *G. excelsum* plants were more than –20%. These reductions might occur because energy produced in the light reactions cannot be used for CO_2 fixation. In water-stressed leaves, the intercellular CO_2 concentration is low because of stomatal closure, and mesophyll resistances likely contribute to further reducing the CO_2 concentration and, consequently, to limiting the CO_2 fixation rates. That leads to a discrepancy between the electron transport rate and carbon fixation rates in the chloroplasts, which results in an increase in the rate of O_2, compared with CO_2, reduction by photosynthetic electron transport (Loreto et al. 2004). Electron flow toward oxygen thus increases, particularly through photorespiration (Cavender-Bares and Bazzaz 2004). As a consequence, electron transport and photochemical efficiency decline with increasing water stress and stomatal closure because the photorespiratory cycle is a less-efficient electron sink than carbon dioxide reduction (Stryer 1988). Our study was consistent with this notion, indicated by the decrease in the performance due to electron transport probability (P_{ET}) in leaves of vulnerable *G. ilicifolium* and common *G. spinigerum* plants. Moreover, when water stress became severe, leaves of these two species may have suffered photodamage, indicated by the increase in the proportion of inactivated RCs or silent

centers (decreased P_{TR}) and faster decrease of Q_A reducing RC/ABS.

The smaller decline in PI in the endangered *G. reticulatum* and rare *G. excelsum* coincided with greater rates of decrease in the two PI components related to absorption and light reactions (RC/ABS and P_{TR}), confirming the lesser degree of damage to PSII. Moreover, plants of these two species re-grew well after the pots were re-watered. Thus, the endangered *G. reticulatum* and rare *G. excelsum* deal with drought conditions by continuing carbon uptake (i.e., requiring continued photosynthetic electron transport). The evidence for this is that the two species maintained higher CO_2 fixation rates during water stress periods (unpublished data). However, other mechanisms which were not investigated here, such as osmotic adjustment or continuing metabolism depending on alternative acceptors for photosynthetic electron transport, may also play important roles.

The results of the water stress experiment provide some explanations for the restricted distribution of the endangered *G. reticulatum*, vulnerable *G. ilicifolium*, and rare *G. excelsum*. The endangered *G. reticulatum* grows on very shallow, rocky soil, which dries out very quickly during the dry season. Therefore, the endangered *G. reticulatum* is shaped by this constraint, necessitating drought tolerance strategies. The vulnerable *G. ilicifolium* was less tolerant of water stress and it often grows close to watercourses where moisture is available. The rare *G. excelsum* showed physiological capabilities for adaptation to water stress and high light conditions that it encounters in its natural vine thicket habitat.

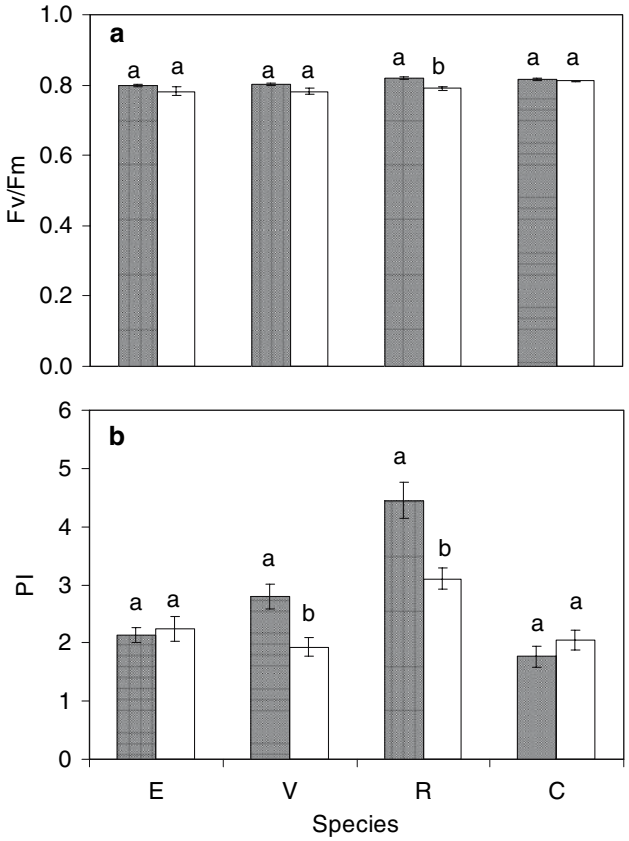

Fig. 8 Fv/Fm (**a**) and PI (**b**) of the four species measured in their natural habitats in summer (▨) and winter (□). For each species, significantly different means (ANOVA, Bonferoni post-hoc test, $P < 0.05$) are indicated with different letters. Values are averages of nine replicates (±SEM)

Natural habitats

The natural habitats of the four species are different in almost all environmental factors. Accordingly, they differ in Fv/Fm and especially PI values. Figure 8a shows that the Fv/Fm ratios of the four species measured in their natural habitats are very similar, except for the rare *G. excelsum*, where this ratio was significantly higher in summer than in winter (0.82 and 0.79, $P < 0.0.5$). Comparing PI of the four species between summer and winter showed that seasonal changes in environmental factors did not affect this value in *G. reticulatum* and *G. spinigerum*, while significant differences ($P < 0.05$) were found in the vulnerable *G. ilicifolium* and rare *G. excelsum* where the values measured in summer were higher.

Seasonal differences in all natural habitats included higher water availability (resulting from higher monthly rainfall), higher temperatures, and higher ambient PAR in summer than in winter (data not shown). The reduction in PI of the vulnerable *G. ilicifolium* in winter seems to be an effect of lower water availability, because this species was vulnerable to water stress. The rare *G. excelsum* may be sensitive to chilling, since this species has shown capacity to tolerate water and light stress. However, it is difficult to determine which factor plays the central role in the changes of PI because the effects of these factors are not independent. Moreover, the PIs in the endangered *G. reticulatum* and common *G. spinigerum* measured in summer and winter were unchanged. An explanation for this observation may be that seasonal differences were not sufficiently strong to cause a significant change in PI.

Conclusions

Differences in conservation status and characteristics of the natural habitats between the four species broadly correspond to the plant physiological responses to the environmental factors tested here. Soil, especially nitrogen and phosphorus limitation, is probably not a factor in the restricted distribution of the endangered *G. reticulatum*, vulnerable *G. ilicifolium*, or rare *G. excelsum*. Sensitivity to

photoinhibition is likely to restrict the endangered *G. reticulatum* to shade habitats, and water availability may determine the location and distribution of the vulnerable *G. ilicifolium* and common *G. spinigerum*. PI emerged as a more sensitive indicator of two environmental stress factors, high light and water stress, than Fv/Fm. The PI and its three components are useful quantitative and non-destructive indicators of plant stress, which can be used in situ to assess plant populations.

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