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Research article

Variegation in *Arum italicum* leaves. A structural–functional study

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ABSTRACT

The presence of pale-green flecks on leaves (speckling) is a frequent character among herbaceous species from shady places and is usually due to local loosening of palisade tissue (air space type of variegation). In the winter-green *Arum italicum* L. (Araceae), dark-green areas of variegated leaf blades are ca. 400 μm thick with a chlorophyll content of 1080 mg m^{-2} and a palisade parenchyma consisting of a double layer of oblong cells. Pale-green areas are 25% thinner, have 26% less chlorophyll and contain a single, loose layer of short palisade cells. Full-green leaves generally present only one compact layer of cylindrical palisade cells and the same pigment content as dark-green sectors, but the leaf blade is 13% thinner. A spongy parenchyma with extensive air space is present in all leaf types. Green cells of all tissues have normal chloroplasts. Assays of photosynthetic activities by chlorophyll fluorescence imaging and O_2 exchange measurements showed that variegated pale-green and dark-green sectors as well as full-green leaves have comparable photosynthetic activities on a leaf area basis at saturating illumination. However, full-green leaves require a higher saturating light with respect to variegated sectors, and pale-green sectors support relatively higher photosynthesis rates on a chlorophyll basis. We conclude that i) variegation in this species depends on number and organization of palisade cell layers and can be defined as a "variable palisade" type, and ii) the variegated habit has no limiting effects on the photosynthetic energy budget of *A. italicum*, consistent with the presence of variegated plants side by side to full-green ones in natural populations.

1. Introduction

Patterns of different green pigmentations on leaves are a frequent occurrence in plants. Leaf variegation may have different origins [1]. Plants with normal green leaves can occasionally develop whitish areas as a consequence of faulty differentiation of chloroplasts due to mutations of nuclear or plastid genes in some or all cell layers [2], leading to impaired photosynthesis [3]. Some of these "chimeral" plant varieties have been developed as garden ornamentals, although they are rarely encountered in nature.

On the other hand, the presence of flecked patterns on leaves (structural variegation or speckling) is a frequent developmental character among herbaceous species living in shady places and forest understorey [4] and is often associated with the enlargement of leaf blades [5]. Variegation may result in a more or less random distribution of dark-green and more lightly pigmented areas, as occurs in the cuckoo pint (*Arum italicum* L.), a member of the Araceae common in Italy. Other European plants, on the other hand, display elaborated, species-specific leaf patterns with individual variants (e.g. species of *Cyclamen* and *Pulmonaria*; *Hepatica nobilis*). The lighter pigmentation of these leaf patches depends, in most instances, on reduction of a continuous palisade tissue to an assemblage of more or less disperse cells with wider intercellular spaces ("air space" type [1]). Tsukaya et al. [6] have described in leaves of *Schismatoglottis calyptrata* (Araceae) a new "air space" variant of variegation based on green palisade cells clustered around special cone-shaped epidermal cells.

Structural leaf speckling occurs in some, but not all, specimens or locations in several undergrowth species, including *A. italicum*. Variegated *Arum* plants can coexist with full-green plants in natural and man-made habitats (moist woods, shady slopes). This fact alone suggests that structural variegation may not be detrimental to fitness in *A. italicum*, although some loss of photosynthetic productivity might be expected. The point remains to be investigated, however, as most studies of variegated leaves were concerned with gross anatomic aspects and have seldom addressed the ecophysiological consequences and correlations of the leaf structure. Some

Abbreviations: AOI, area(s) of interest; Carot, total carotenoids; Chl, total chlorophyll; ETR, linear photosynthetic electron transport rate; NPQ, non-photochemical quenching; PAM, pulse amplitude modulated; PSII, photosystem II; TEM, transmission electron microscope.

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photosynthetic features of *A. italicum* have been studied in green leaves [7], but despite the interesting ecophysiological properties of this species a comparison of full-green and variegated leaves has never been done.

We have examined *A. italicum* leaves by light- and electron microscope investigations combined with analyses of chloroplast pigments and photosynthetic parameters. The results indicate a strong developmental variability as the structural basis of *Arum* leaf variegation, which essentially depends on the number and organization of palisade cell layers ("variable palisade" type of variegation). They also reveal a surprising physiological regulation of variegated and full-green leaves, resulting in uniform photosynthetic performances although the responses of different leaf sectors at changing light regimes are appreciably different. These observations are entirely consistent with the abundance and persistence of variegation in this species.

2. Results

2.1. Cellular features

Arum italicum plants within a population can be uniformly green, or they show leaf variegation with sharply defined sectors of two or more shades of green colour (Fig. 1A,B). We have investigated the tissue organization of the flecks by light microscopy on thin sections of variegated leaf blades containing areas with two grades of pigmentation, "pale-green" or "dark-green" (Fig. 1B). The study revealed that palisade tissue of dark-green sectors consisted of two compact tiers of elongated cylindrical cells (Fig. 2A), 170 μm thick (Table 1). In pale-green sectors of the same leaf the palisade was reduced to a single, loose layer of halved thickness consisting of small, stunted cells (Fig. 2B). On the other hand, the spongy tissue

Fig. 1. (A) *Arum italicum* plant with variegated leaves. (B) Detail of a speckled leaf.

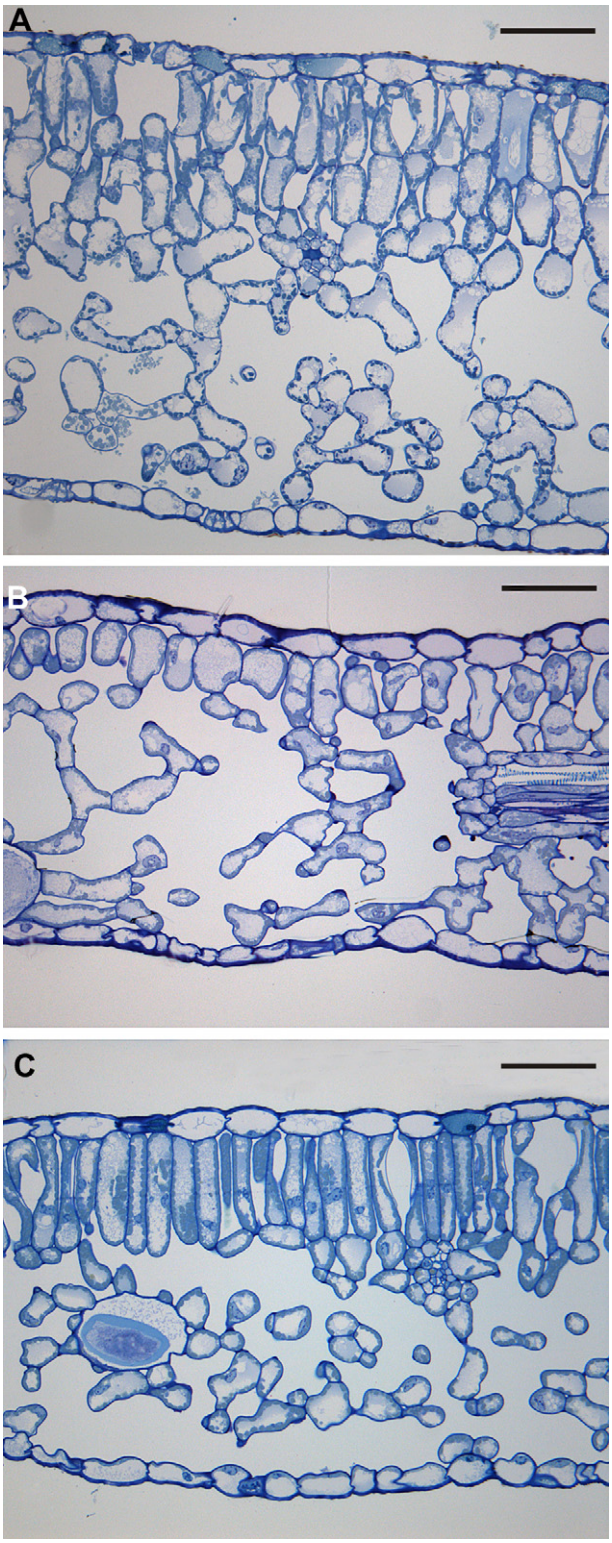

Fig. 2. Thin cross-sections of a dark- (A) and a pale-pigmented area (B) from a variegated leaf blade and (C) from a full-green leaf blade (bar = 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Mean thickness of tissues (±sd) in dark- and pale-green areas of variegated or full-green leaves of *A. italicum*.

	Leaf thickness (μm)	Palisade tissue thickness (μm)	Spongy tissue thickness (μm)
Dark-green areas	406 ± 24	173 ± 13	229 ± 15[a]
Pale-green areas	304 ± 25	85 ± 12	219 ± 19[a]
Full-green leaf	353 ± 22	134 ± 12	219 ± 19[a]

Values followed by *a* superscript are not significantly different ($P < 0.05$) as determined by DMTR, values without superscript are significantly different ($P < 0.05$).

consisted of cells surrounded by a wide air space and had a constant thickness of about 220 μm, without obvious morphological variations in different leaf areas. In fact, leaf thickness was ca. 400 μm in dark-green variegated sectors, whereas pale-green sectors were ca. 300 μm thick (Table 1). The variable thickness and arrangement of the palisade tissue in differently pigmented regions was evident in fresh hand-cut sections of speckled leaf blades (Fig. 3A,B), which also showed that all palisade cells, regardless of the tissue structure, contained a full set of green chloroplasts.

On the other hand, full-green *Arum* leaves (Figs. 2C and 3C) exhibited a palisade tissue with a single, closed array of slender cylindrical cells packed with chloroplasts. The average thickness of the palisade in these green leaves was significantly lower (−23%) than in variegated dark-green areas. As a consequence, these leaves were 13% thinner than dark-green sectors of spekled leaves (Table 1). The anatomical organization of full-green leaves thus appears to be intermediate between those of pale- and dark-green areas of speckled leaves.

2.2. Chloroplast ultrastructure

Chloroplast ultrastructure was examined in *Arum* leaf tissues by TEM in a search for possible alterations of organelles in weakly pigmented areas. No relevant differences could be found between chloroplasts of different leaf regions, however. Chloroplasts of palisade cells in dark-green areas (Fig. 4A) and pale-green areas (Fig. 4B) and of the spongy tissue (Fig. 4C,D) looked normal, with a well-developed thylakoid system containing abundant grana. Similarly, ultrastructural investigation of mesophyll cells of full-green leaves confirmed the presence of normally organized chloroplasts (not shown).

2.3. Photosynthetic pigments and photosynthetic activity

A study of the photosynthetic capabilities of differently pigmented areas of *A. italicum* leaves was performed by measuring chloroplast pigments and functional parameters related to photosynthesis in selected areas of the leaves. The contents of chlorophyll (1080 mg m^{-2}) and carotenoids (178 mg m^{-2}) were similar in full-green leaves and variegated dark-green sectors on a leaf area basis (Table 2) despite their different structures and thicknesses, suggesting some form of regulatory compensation. In variegated pale-green sectors the amounts of chlorophylls (−26%) and carotenoids (−25%) per surface unit were appreciably lower than in full-green sectors, but chl *a*/chl *b* ratios and carot/chl ratios were almost unchanged (Table 2). The decreased contents of photosynthetic pigments in these lighter areas were clearly related to the reduced stratification and size of palisade cells.

As a measure of photosynthetic function, the main parameters linked to chl fluorescence emission in vivo were assayed in small, selected areas of interest (AOIs) on leaves by PAM imaging (Fig. 5). The analyses showed that Fv/Fm ratios rose to a constant value of

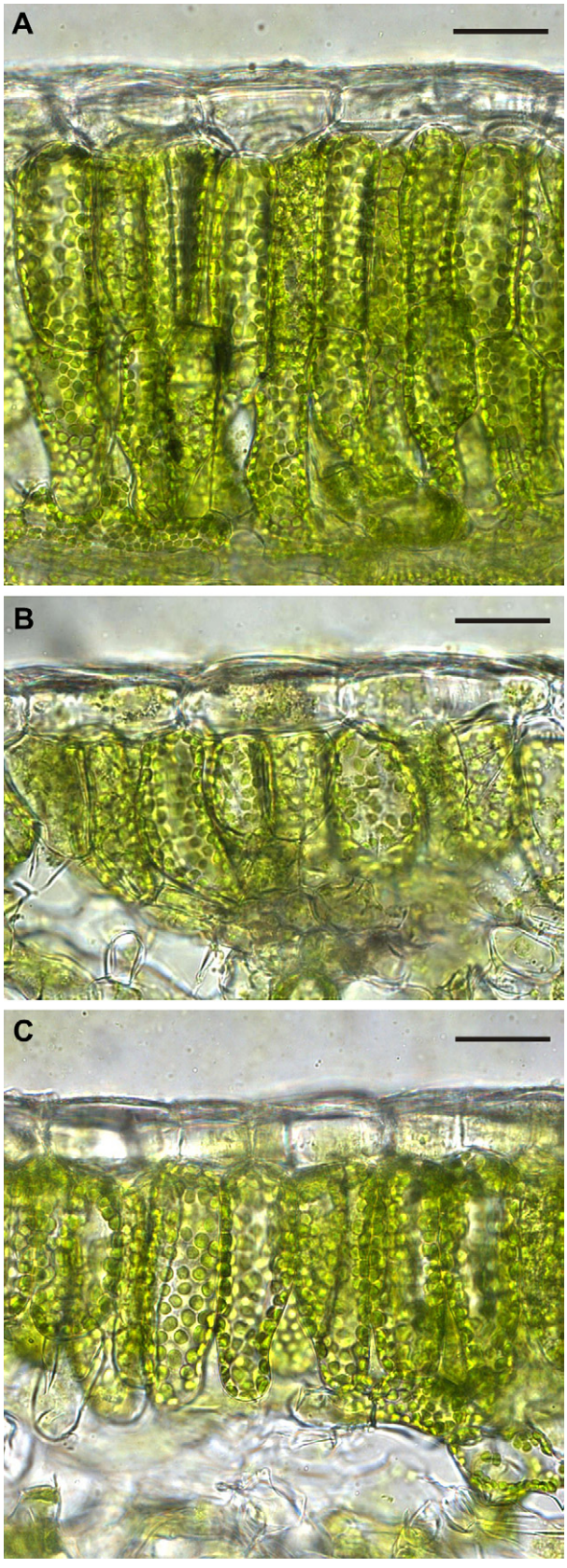

Fig. 3. Hand made transversal sections of palisade tissues from a dark- (A) and a pale-pigmented area (B) of a fresh variegated leaf blade and (C) of a fresh full-green leaf blade (bar = 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

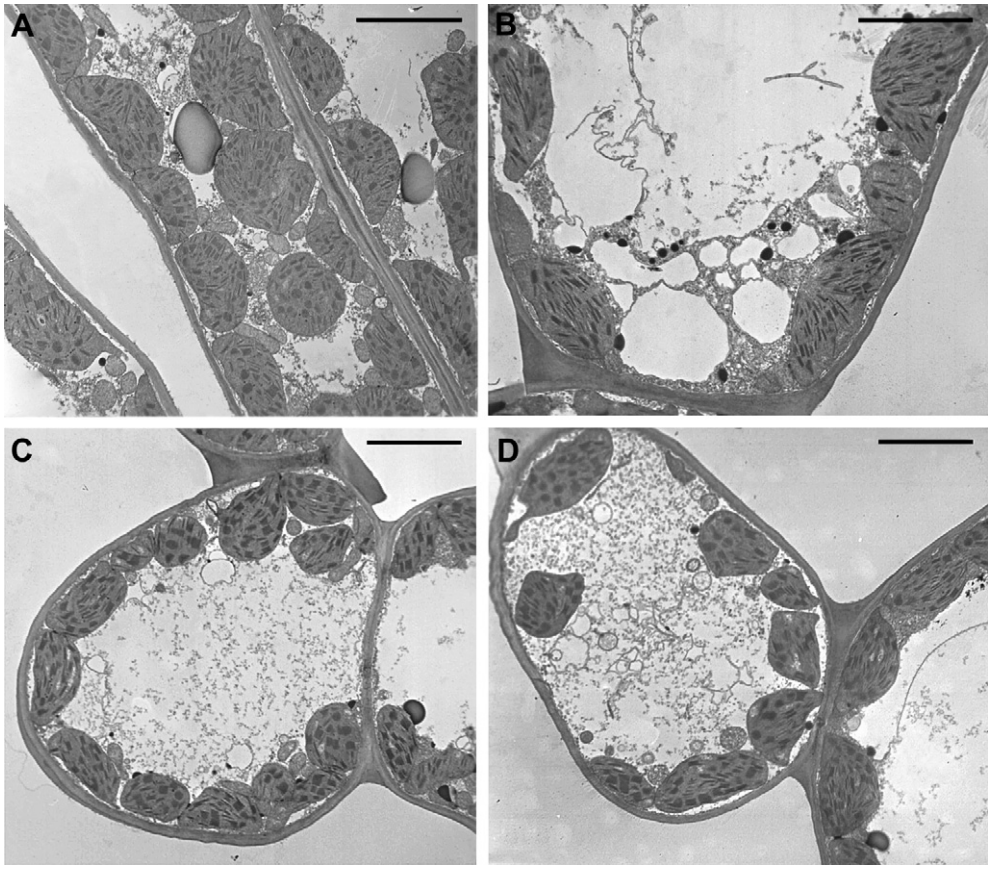

Fig. 4. Micrographs at the transmission electron microscope of a variegated leaf. Palisade cells from a dark- (A) and from a pale-green region (B). Spongy cells from a dark- (C) and from a pale- (D) green region. The chloroplast organization is the same in the differently pigmented areas. (bar = 5 μm).

0.79–0.80 in both dark- and pale-green areas of variegated leaves as well as full-green leaves. Such high values are typical of chloroplasts with efficient PSII function [8] and support the absence of photosynthetic alterations in green cells in pale-green areas. Fluorescence-derived electron transport rate (ETR) curves at increasing light irradiance (Fig. 6A), studied in different regions of variegated leaves of *A. italicum*, were indicative of relatively shade-adapted plants and were overlapped at low light intensity. ETR in both pale- and dark-green areas attained half-saturation at a PAR irradiance of 80 μmol photons m^{-2} s^{-1} and full light-saturation at about 300 μmol photons m^{-2} s^{-1}. At this saturating irradiance the ETR values of pale-green areas were 14% superior to dark-green ones on an AOI absorptivity basis (and, consequently, with reference to variable amounts of cells and pigments in each AOI). The effective quantum yields of PSII (ΦPSII) were indistinguishable in differently pigmented sectors of variegated leaves when measured under nonsaturating light (up to 100 μmol photons m^{-2} s^{-1}, Fig. 6B).

Table 2
Total chlorophyll and carotenoids per leaf area, chl *a*/*b* and carot/chl ratios (±sd) in dark- and pale-green areas of variegated or full-green leaves of *A. italicum*.

	mg chl($a + b$) m^{-2}	mg carot m^{-2}	chl *a*/*b*	carot/chl
Dark-green areas	1080 ± 50[a]	178 ± 6[a]	2.7 ± 0.1[a]	0.16 ± 0.01[a]
Pale-green areas	800 ± 20	134 ± 5	2.9 ± 0.2[a]	0.17 ± 0.01[a]
Full-green leaf	1110 ± 60[a]	184 ± 11[a]	2.7 ± 0.1[a]	0.17 ± 0.02[a]

Values followed by *a* superscript are not significantly different ($P < 0.05$) as determined by DMTR, values without superscript are significantly different ($P < 0.05$).

On the other hand, full-green leaves were half-saturated at about 130 μmol photons m^{-2} s^{-1} and barely light-saturated at the maximum light output by the PAM instrument, i.e. 460 μmol photons m^{-2} s^{-1} (Fig. 6A), and their ΦPSII values under non-saturating light were lower (Fig. 6B). The common decline of effective quantum yield ΦPSII at increasing light intensities is clearly discernible in Fig. 6B, which also emphasizes the decreased photosynthetic efficiency of dark-green sectors and full-green leaves at irradiances >100 m^{-2} s^{-1} in striking contrast with the performance of pale-green sectors. Indeed, ΦPSII values of dark-green and full-green tissues tend to converge at elevated irradiances, while ΦPSII values of pale-green sectors are always higher (Fig. 6B). Finally, plots in Fig. 6C show non-photochemical quenching (NPQ) as a measure of light energy dissipation in the same experiments, confirming that full-green leaves release back a remarkable fraction of radiant energy absorbed in low light (+180% compared to variegated leaves at 100 μmol photons m^{-2} s^{-1}).

Further information on the regulation of photosynthesis in different areas of variegated plants was gained from measurements of photosynthetic O_2 evolution under fully saturating light by small disks obtained from leaf blades. The absolute photosynthesis rates expressed on a leaf area basis (Table 3) were comparable between various types of green areas and only marginally lower in pale-green sectors. Rates expressed on a chl basis, in fact, were distinctly higher (+28%) for pale-green compared to dark-green sectors or full-green leaves, which gave similar values. All experiments thus demonstrate that pale-green sectors can conduct unimpaired photosynthetic activity under most conditions and may be relatively more efficient under some conditions.

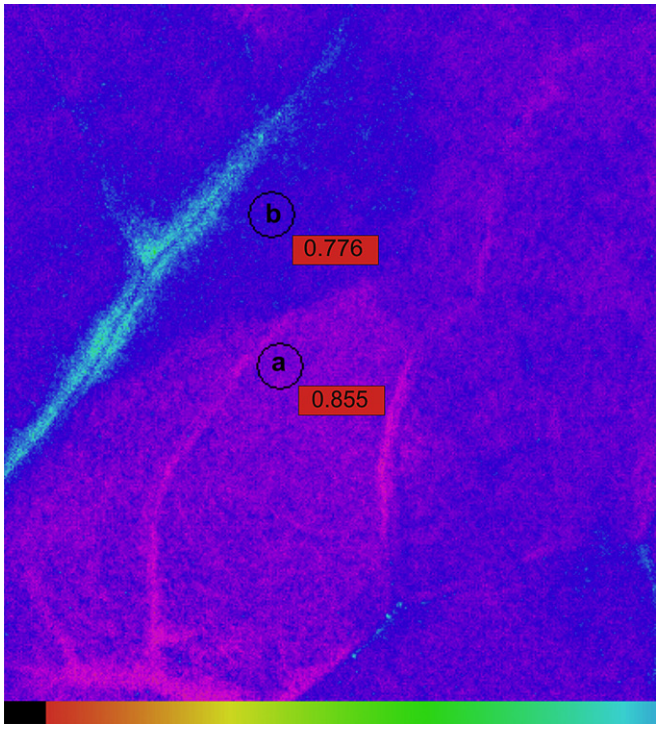

Fig. 5. Image captured by CCD-camera of imaging PAM showing the absorptivity values of two areas of interest of 4 mm diameter selected from a dark- *(a)* and a pale-green *(b)* area of a variegated leaf. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Discussion

3.1. Leaf morphology

We have studied the leaf tissue structure and photosynthetic parameters of *A. italicum* (cuckoo pint), a common south-European plant which presents either variegated (speckled) leaves with varying numbers of pale-green areas, or uniform green leaves. The morphology of these variants is stable in the course of years, and speckled plants often occur together with full-green ones in mixed populations. Inspection of a large number of specimens of both types leads us to conclude, in the first place, that variegation in *A. italicum* does not affect chloroplast differentiation or ultrastructure. In fact, chloroplasts viewed at TEM are well-organized and similar in all types and areas of *Arum* leaf blades, either in pale- or dark-green areas, in palisade as well as in spongy parenchyma. Second, the variable leaf pigmentation of speckled plants (Fig. 1B) appears to depend on the structure of the palisade chlorenchyma which consists of a single rather loose layer of short, plump cells in pale-green sectors but of a double layer of compact, elongated cells in dark-green ones. The developmental choice between pale- and dark-green areas in the speckled leaf of *A. italicum* thus entails an additional regulation of the proliferation and cross-divisions of palisade cells. This is probably a simplified scheme, since some variegated plants actually display a mosaic of leaf sectors with various shades of green colour at a closer glance, but it is likely that the present arguments could also apply, in principle, to multi-sectored *Arum* plants. Notably, the thickness of the uniseriate palisade of full-green leaves is exactly halfway between pale- and dark-green areas of variegated plants, as is leaf thickness itself (Table 1).

The dark-green/pale-green patchwork of *A. italicum* leaves can be ascribed to an "air space" type [1] of tissue in a wide sense, but the term as applied here may be misleading. The coexistence of leaf sectors with one or more palisade cell layers we see in *Arum* is an

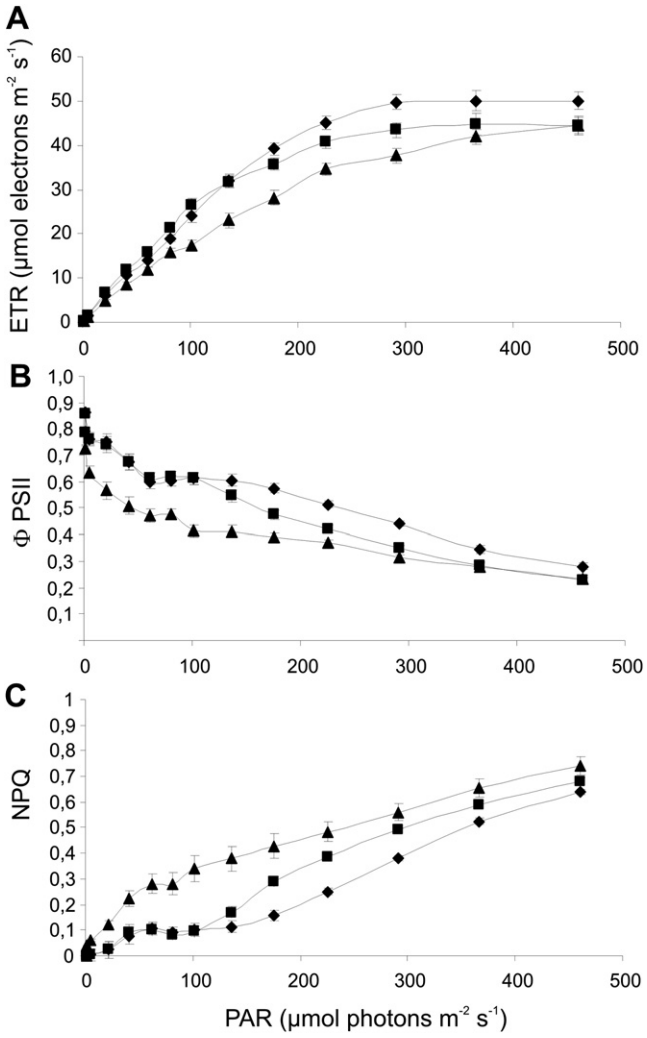

Fig. 6. (A) Electron transport rate (ETR) of pale- (◆) and dark- (■) green areas from a variegated leaf and of a full-green leaf (▲) at increasing PAR intensities. Calculated from imaging PAM fluorescence data, on AOI absorptivity basis. (B) PSII quantum yield (ΦPSII) and (C) non-photochemical quenching (NPQ) in the three types of leaf areas, derived from the same set of data.

unusual situation, different from the types of air space variegation reported for other plants (including quite a few of the Araceae family), mostly based on a scattered distribution of green cells in a single palisade cell tier [1] or, less commonly, on discrete clusters of palisade cells [6]. A partial disruption of multiseriate palisade parenchyma to give way to lighter areas has recently been reported by Konoplyova et al. for *Cyclamen* species [9] and may be of wider occurrence. These authors [9] see, however, a single palisade cell layer in *Arum* leaves, dark-green sectors included (where we consistently find two), and estimate a sizeable widening of air space in pale-green leaf sectors. The latter conclusion appears unlikely,

Table 3
Photosynthetic oxygen output (±sd) by leaf disks from differently pigmented areas or full-green *Arum* leaves under saturating PAR illumination, referred both to surface area (m^2) and mg chl.

	µmol O_2 m^{-2} s^{-1}	µmol O_2 (mg chl)$^{-1}$ h^{-1}
Dark-green areas	7.5 ± 0.4^a	25 ± 2^a
Pale-green areas	7.1 ± 0.5^b	32 ± 3^b
Full-green leaf	7.3 ± 0.3^{ab}	24 ± 2^a

Values followed by the same letters were not significantly different ($P < 0.05$) as determined by DMTR.

since the reduced thickness of pale-green sectors in the presence of an invariant spongy parenchyma (see Table 1) is clearly inconsistent with a substantial expansion of internal leaf volume and air spaces. We conclude that leaf speckling in *A. italicum* represents a new type of variegation primarily depending on the variable thickness and cell organization of the palisade tissue, and it could more aptly be defined as a "variable palisade" type.

3.2. Photosynthetic features

As was pointed out for other Araceae [6], the stable coexistence of speckled and full-green forms in *A. italicum* suggests itself that leaf speckling is probably not selected against. Lee [5] provided evidence that variegation may be beneficial to photosynthesis in rain forest understorey plants, and Konoplyova et al. [9] found only minor differences in photosynthetic CO_2 uptake between pale- and dark-green sectors of *Cyclamen* leaves, paler areas even being superior in some respects. The presence of whitish flecks on leaves of *A. italicum* does not appear to have limiting effects on the energy budget of speckled plants, since all green cells in these lighter areas contain functional chloroplasts and the lower amount of pigments per unit surface is compensated by higher relative photosynthetic electron transport rates at high light intensity (see Fig. 6A). This contention is supported by O_2 exchange measurements in saturating light which also yield comparable values for both types of areas (Table 3). Improved light paths throughout the inside of the leaf and down to the spongy parenchyma may be a relevant factor for the good photosynthetic performances of speckled *Arum* plants, with a likely functional involvement of the double palisade layer of green sectors especially under limiting light thanks to enlarged cell surfaces and facilitated gas exchange. Pale-green and dark-green sectors together thus seem to ensure competitiveness to variegated leaves of *A. italicum*.

Full-green leaves, on the other hand, show reduced PSII yield, higher NPQ and significantly lower ETR under nonsaturating light with respect both to pale-green and dark-green areas of speckled leaves (Fig. 6), although they resemble the latter ones in terms of chloroplast pigments and maximum photosynthetic activity (Table 3). Full-green *A. italicum* leaves seem, in fact, to be less shade-tolerating than variegated ones, possibly in relation to their uniseriate palisade tissue. This fact may be unfavourable in the weak light under a forest canopy, but it should be considered that such environments are not uniformly illuminated and are erratically subject to light beams of variable intensities [10,11]. The range of photosynthetic responses displayed by different *Arum* tissues as reported above may improve the exploitation of the understorey light, from limiting to strong, sunflecks in particular.

3.3. Ecological benefits

Taken now for granted that leaf speckling with pale-green and dark-green sectors is not detrimental to photosynthesis in *A. italicum* and other species, the question arises as to whether it does also confer some kind of additional benefit. The widespread occurrence of variegated leaves among understorey herbs of Europe (notably *Cyclamen* spp., *H. nobilis* and some orchids, in addition to *Arum*) suggests that leaf speckling may be ecologically favourable to these plants. In some instances (e.g. *Pulmonaria* spp., *Erythronium*) the colour pattern is even subject to change with seasons. Some evidence exists that leaf variegation may be useful to forest plants to escape herbivory and phytophagous insects by mimicry or other ingenious defence devices [4,12–15] and this might be the case for *A. italicum*, too. Similarly, the gaudy variegation of many thorny plants in semi-arid areas has been proposed to have an aposematic (warning) function towards ungulates [16]. However, the underlying advantage mechanism of leaf speckling does not need to be always one and the same. Some flecked patterns might represent, for example, a direct deterrent for herbivores or a visual signal for pollinators, though the latter possibility is unlikely for *A. italicum* which allures insects by emitting odour [17,18]. All in all, much remains to be understood in the wide field of plant variegation [19].

4. Materials and methods

4.1. Plant material

Analyses were carried out on variegated or uniformly green plants of *A. italicum* L., obtained from hills near the city of Bologna and grown for several years in the Botanic Garden of Bologna University on manured ground in the open. Fully expanded leaves of comparable age and size were sampled during autumn months in years 2007–2009. Although some specimens exhibit three or more shades of increasingly lighter flecks, most variegated leaves have only one type of pale-green areas between veins and we have always used the latter plants. In the following, different sectors of variegated leaves will be referred to as "pale-green" and "dark-green" areas, and uniformly green leaves will be called "full-green".

4.2. Light and electron microscopy

Tissue samples from dark- and pale-green areas of variegates leaves and full-green leaves were fixed overnight at 4 °C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9), postfixed for 2 h in 1% osmium tetroxide in the same buffer and then processed as described by Rascio et al. [20]. For TEM, ultrathin sections (800 nm) were cut with an ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria), stained with lead citrate and examined with a transmission electron microscope (TEM 300, Hitachi, Tokyo, Japan) operating at 75 kV. For light microscopy thin sections (1 μm) cut with the same ultramicrotome were stained with equal volumes of 1% toluidine blue and 1% sodium tetraborate and examined under a light microscope (Ortholux, Leitz-Wetzlar, Germany). The same microscope was used to inspect hand-cut leaf sections. On these sections 50 measurements of leaf blade and palisade thickness for each differently pigmented area were carried out.

4.3. Chlorophyll and carotenoid determination

Chlorophylls and total carotenoids in dark-green and pale-green areas of variegated leaves were analysed in a double-beam spectrophotometer (GBC UV/VIS 918, GBC Scientific Equipment Pty Ltd., Victoria, Australia) after extraction with N,N-dimethylformamide, and pigment concentrations were calculated using the extinction coefficients of Porra et al. [21]. Analyses were repeated six-times on as many independent samples. Similar samples were also used for carot analysis by reversed-phase HPLC, according to La Rocca et al. [22]. The tests were replicated 3-times.

4.4. Assay of in vivo oxygen release

Photosynthetic oxygen release was measured as previously described [23] on small disks of dark-green and pale-green tissues and full-green leaves, according to Ishii et al. [24] using an oxygen monitor (YSI, Model 53, Yellow Springs Instruments Co., OH, USA). The tests were replicated 6-times on as many independent samples.

4.5. PAM imaging and fluorescence parameters

In vivo chl *a* fluorescence of *A. italicum* leaves was measured at room temperature (22 °C) with fluorometer Imaging PAM 2000

(Walz, Effeltrich, Germany). This system allowed to perform non-invasive and accurate determinations of chl fluorescence parameters in variously pigmented tissues of a single, intact leaf. The instrument was provided with lights placed in a ring arrangement and directed at fixed angle and distance onto the leaf area of choice. Two outer LED-rings (in a total of 96 LEDs) provided the measuring light, actinic light and saturating pulse with peak wavelength at 470 nm. The inner ring of 16 LEDs provided the pulse-modulated light for assessment of absorptivity at 650 nm and 780 nm. The charge-coupled device (CCD) camera had a resolution of 640 × 480 pixels. Pixel value images of the fluorescence parameters are displayed using a false colour code ranging from black through red, yellow, green, blue to pink (0.000–1.000). All measurements were carried out at maximum distance between camera and leaf, the latter blocked with a clip (26 × 34 mm area). Chl *a* fluorescence determinations were obtained from at least 4 variegated or full-green, fully expanded leaves of *A. italicum*. To assess spatial heterogeneity in photosynthetic functions related to different tissue pigmentations, 4 areas of interest (AOIs) of 4 mm diameter from differently pigmented regions were selected on each leaf on the basis of tissue absorptivity (see Fig. 5). Chl *a* fluorescence measurements were always taken around midday to minimize diurnal fluctuations. The leaves were darkened for 20 min before initiating measurements, except in experiments requiring light adaptation.

Minimum (dark) fluorescence Fo was obtained by applying measuring light pulses at low frequency (1 Hz). Maximum fluorescence Fm was determined by applying an 800 ms saturating (6000 μmol photons m^{-2} s^{-1}) blue pulse (10 Hz). The maximum quantum yield of PSII photochemistry, the Fv/Fm ratio, was determined as (Fm − Fo)/Fm. Values of effective quantum yield of PSII photochemistry, ΦPSII, and of non-photochemical quenching, NPQ, were measured at different light intensities and calculated as: ΦPSII = (F'm − Fs)/F'm; NPQ = (Fm − F'm)/F'm. The calculated electron transport rate (ETR = ΦPSII × PAR × 0.5 × Absorptivity), which assumes equal distribution of PAR radiation between both photosystems, was also measured at different light intensities. The specific absorptivity automatically recorded by PAM imaging for each AOI was considered for ETR calculation.

4.6. Statistical analysis

A one-way analysis of variance (ANOVA) was applied to the data. Statistical analyses were performed with SPSS 10.0 [25]. All probabilities are two tailed. Data were checked for normality and homogeneity of variance (Levene test). Differences between means were evaluated for significance by using Duncan's multiple range test (DMRT) ($P < 0.05$).

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