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Seasonal fluctuations of photosynthetic pigments content in *Taxus baccata* needles

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Abstract: Yew is a coniferous, evergreen, dioecious species. The objective of the study was to present a comprehensive characteristic of changes occurring throughout the year in terms of the content of photosynthetic pigments and related compounds in the needles of yew, depending on the sex of individuals and age of needles. Eight compounds, particularly chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), carotenoids (Car), protoporphyrin IX (PPIX), magnesium protoporphyrin IX (MgPPIX), protochlorophyllide (Pchlide), chlorophyllide a (Chlide *a*), and chlorophyllide b (Chlide *b*), were subjected to quantitative analysis. Based on the several parameters under study, significant differences between male and female individuals were observed, while most commonly, the largest differences were reported in the autumn and winter period. They were related to the content of Chl *a*, Chl *b* and Chl/Car ratio. The remaining compounds showed no significant differences according to the sex and were slightly different only in single periods. For all the studied parameters except for Chl/Car ratio, interaction between sampling dates and sex was not statistically significant. Significant differences between the needles of different age were observed only in terms of the content of Chl *b*, MgPPIX, Pchlide, Chlide *b*, and Chl *a/b* ratio, and these differences were always caused by the current-year needles.

Keywords: chlorophyll, chlorophyllide, needles age, pigments, sex

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Introduction

Yew is a coniferous, good shade-tolerant, dioecious, long-living, and relatively late maturing species (Garcia et al., 2000; Thomas & Polwart, 2003). As long as the generative organs of reproduction do not appear, it is not possible to determine the sex of a particular individuals; studies on sex differences in yew thus face many difficulties. A detailed description of the biochemical and physiological differences occurring between male and female individuals constitutes the first step in the determination of sex markers. Many studies show that dioecious species are more prone to extinction, which is caused by two reasons. Firstly, the necessity of the occurrence of an appropriate number of male and female individuals and secondly the uneven reproductive effort made by both sexes, which directly restricts the vegetative growth in females (De Jong & Van Der Meijden, 2004; Vamosi & Vamosi, 2005; Iszkuło et al., 2009, 2011). As a result of global climate changes, the risk of dioecious species also increases, especially when a given sex exhibits greater habitat requirements (De Jong & Van Der Maijden, 2004; Iszkuło et al., 2009). Additionally, in terms of European yew both, excessive acquisition of raw materials and biological characteristics of the species have caused that in many European countries, its range was limited to few and fragmented population. It has led to recognize this species as endangered and to subject it for protection (Peterken, 1996; Seidling, 1999; Svenning & Magård, 1999; Boratyński et al., 2001; Iszkuło & Boratyński, 2005).

Upon entering into generative reproduction stage, needs of male and female organisms change. Both male and female individuals when reaching sexual maturity and entering into the stage of generative reproduction begin to exhibit physiological differences caused by their reproductive effort. Male flowers of *Taxus baccata* begin to develop in the summer during the year preceding pollination. In the autumn or late winter, production of pollen is observed, which is spread by the wind in February and March, followed by pollination. From the pollination to fertilization, 6–12 weeks elapse. Fertilization occurs between May and June, and from that moment, growth and development of the embryo occurs, maturation of arils and seeds is noticed, which ends in September. At the same time, from May to June, continuous development of female "flowers" happen, which will be pollinated the next year (Hejnowicz, 1978) (Fig. 1). All changes in the physiology of male and female organisms may be observed by the content of biochemical compounds (e.g., pigments, proteins, carbohydrates, enzymatic activity) (Nicotra, 1999; Obeso & Retuerto, 2002; Leigh et al., 2006; Montesinos et al., 2006; Iszkuło et al., 2011).

Previous studies determining the sex of yew were relatively few and were conducted in a limited range. It has been observed that in *T. baccata*, male individuals tend to be larger and have higher annual growth in comparison to female individuals (Lloyd & Webb, 1977). Whereas, needles derived from female individuals are characterized by its higher number and higher density of stomata (Iszkuło et al., 2009). There are also differences in the production of adventitious roots from cuttings collected from male and female *T. baccata* individuals depending on the type of auxin used; however, the mechanism of this reaction is not entirely clear (Nandi et al., 1996). Studies on carbon and nitrogen content have demonstrated the presence of comparable concentrations in both sexes in *T. baccata* (Iszkuło et al., 2009), similarly the taxol content depending on the age and sex of *T. wallichiana* from the Himalayan did not differ significantly between both the sexes (Nadeem et al., 2002). In contrast, Iszkuło et al. (2013) found that female individuals of *T. baccata* exhibited a significantly higher level of taxanes. Female individuals of *T. baccata* are also more demanding in terms of fertility and humidity of the soil (Cedro & Iszkuło, 2011).

The occurrence of biochemical differences occurring in the needles of different life span, related to the processes of development and aging of needles is a different study. In deciduous trees, the process of both synthesis and degradation of pigments is restricted to a single vegetative season. In evergreen trees, the process is extended in time, so most commonly, needles of different age occur on the tree. As for yews, the life span of needles is dependent on the environmental conditions, from 4 to 8 years (Bartkowiak et al., 1978). The progressive aging of the needles eventually leads to an imbalance between biosynthesis and degradation of chlorophyll and may be observed, among others, through changes in the concentrations of pigments and their degradation products (Kutík, 1998; La Porta et al., 2006). The amount of photosynthetic pigments in trees is subjected to changes during growth and development; it also depends, among others, on their age, position around the crown, light conditions, and access to minerals and water (Merzlyak et al., 1999; Silkina & Vinokurova, 2009; Sarala & Saravana, 2011). As the amount of

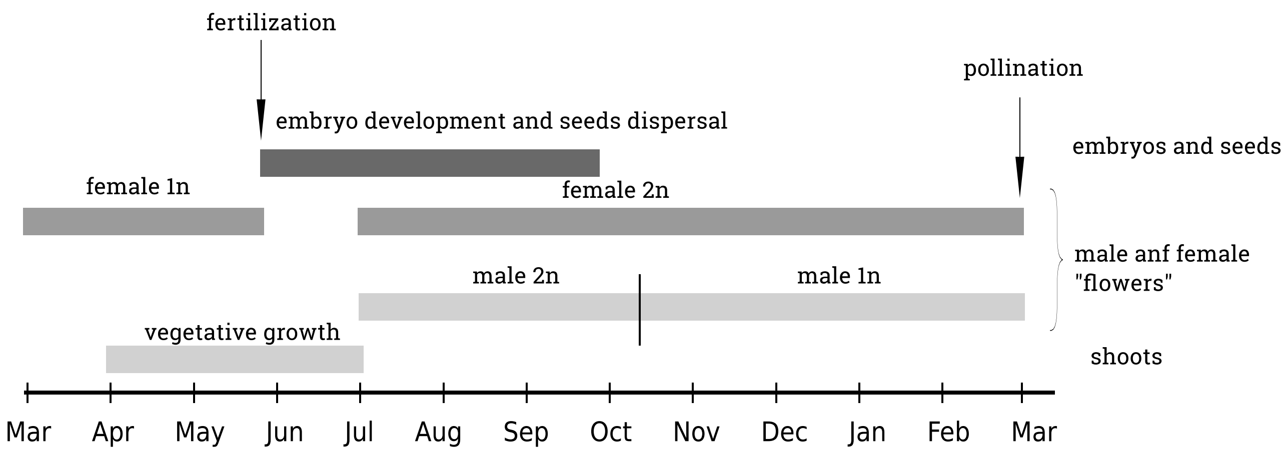

Fig. 1. Diagram of yew generative and vegetative organs annual development

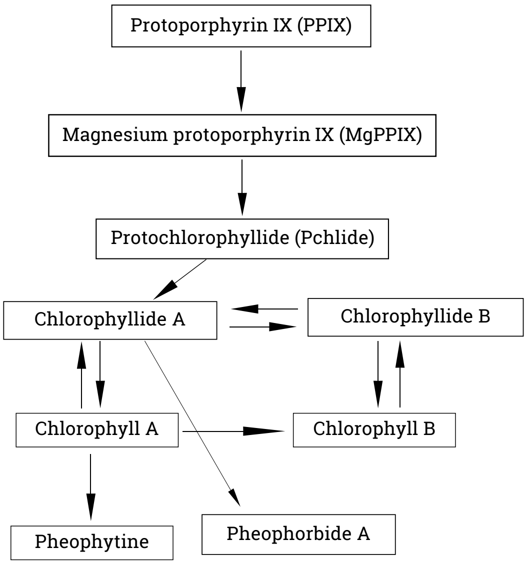

Fig. 2. The partial pathways of chlorophyll biosynthesis and degradation based on compounds used in this research

light absorbed by the plant is based on the amount of photosynthetic pigments, it can be understood that chlorophyll content has a direct impact on the photosynthetic potential and primary production.

Chlorophyll biosynthesis is a very complex process which involves over 50 biochemical reactions during the synthesis from precursors through, among others, protoporphyrin IX (PPIX), magnesium protoporphyrin IX (MgPPIX), protochlorophyllide (Pchlide), chlorophyllide a (Chlide *a*) and chlorophyllide b (Chlide *b*), and ending with chlorophyll degradation to pheophytin and through pheophorbide to low-molecular weight degradation products (Fig. 2) (Von Wettstein et al., 1995; Yang et al., 1998).

The main objective of the study was to determine differences in the content of photosynthetic pigments and their selected precursors and degradation products between female and male individuals of yew and to determine the course of changes over one year. Additionally, the effect of needles age on the selected parameters of photosynthetic apparatus was investigated.

Materials and methods

Plant material

The study had been conducted for 12 months from March 2013 to February 2014. In the Botanical Garden of the Jagiellonian University in Krakow, 18 European yew individuals (including 12 female and 6 male individuals) grown under similar light conditions were selected. Each of the individual selected was precisely identified in terms of sex by repeated observations of the occurrence of visible generative reproduction organs: male "flowers" and seeds in arils or the remaining arils in female individuals. Collecting of biological material in the form of shoots with needles was conducted regularly every month, separately for each individual. To minimize the effect of changeable light conditions on the results, shoots were evenly collected from the entire perimeter of the crown of each individual, from its outer part at a height between 1 and 2 m, and then placed in the container on ice. After transportation to the laboratory, shoots were categorized based on its annual increment and classified into one of four groups as: 0 annual – current-year-old needles, 1 annual – one-year-old needles, 2 annual – two-year-olds needles and 3 annual – three-year-olds needles. Then, needles were collected followed by their further homogenization in liquid nitrogen and freeze-drying (Labconco FreeZone 2.5 Liter Freeze Dry System, Kansas City, USA). Material protected in such a way was stored until analysis.

Extraction and quantification

Absorbance analysis was performed using Helios Gamma spectrophotometer (Thermo Spectronic, Cambridge, UK). Quantitative analysis was conducted for eight compounds: Chl *a* and Chl *b* according to PORRA et al. (1989), Car according to Lichtenthaler (1987), Chlide *a* and Chlide *b* according to McFeeters et al. (1971), Pchlide, PPIX, and MgPPIX according to Kahn et al. (1976). The extraction was performed by using 50 mg of freeze-dried powder with 4 ml of 80% buffered acetone. In order to determine Chl *a*, Chl *b*, and Car, the measure of absorbance was performed directly from the buffered acetone after appropriate dilution of the samples. Other compounds were determined after purification of samples with hexane (1:1 v/v). Based on the measurements obtained, the ratio of Chl *a/b* and Chl/Car were calculated.

Statistical analysis

In order to determine the significant differences in the content of the compounds determined, in each of the tested periods, repeated measures of ANOVA (RM-ANOVA) was used. Tukey's test was used as a post-hoc test for different *N*. In terms of evaluation of the differences between sexes, N for each term was established at N = 24 for male and N = 48 for female individuals. Assessing differences between sexes, samples from different year-old needles were combined in groups for particular individuals. In studies regarding differences between needles age in particular sampling dates, N was established at 18 for each year investigated (no division into male and female individuals was made). Homogeneity of variance was tested with Bartlett's test (Sokal & Rohlf, 1995). For the data which did not meet the ANOVA assumptions, data transformation or at least ANOVA Kruskal–Wallis non-parametric test was performed. All analyzes were performed using two grouping variables: sex (male and female – without dividing needle's age) and age of needles (current-year-old, one-year-old, two-years-old, and three-years-old – without dividing sex). Annuals of needles were compared from the moment of current year's increment, that is, within the period from May to February. All statistical analyzes were conducted using Statistica software, version 10.0 (StatSoft Inc., Tulsa, OK, USA). Average values are presented as the mean ± one standard error of the mean (SE). Statistical significance was established as p≤0.05.

Results

Sex differences

For several investigated parameters, significant differences between male and female individuals

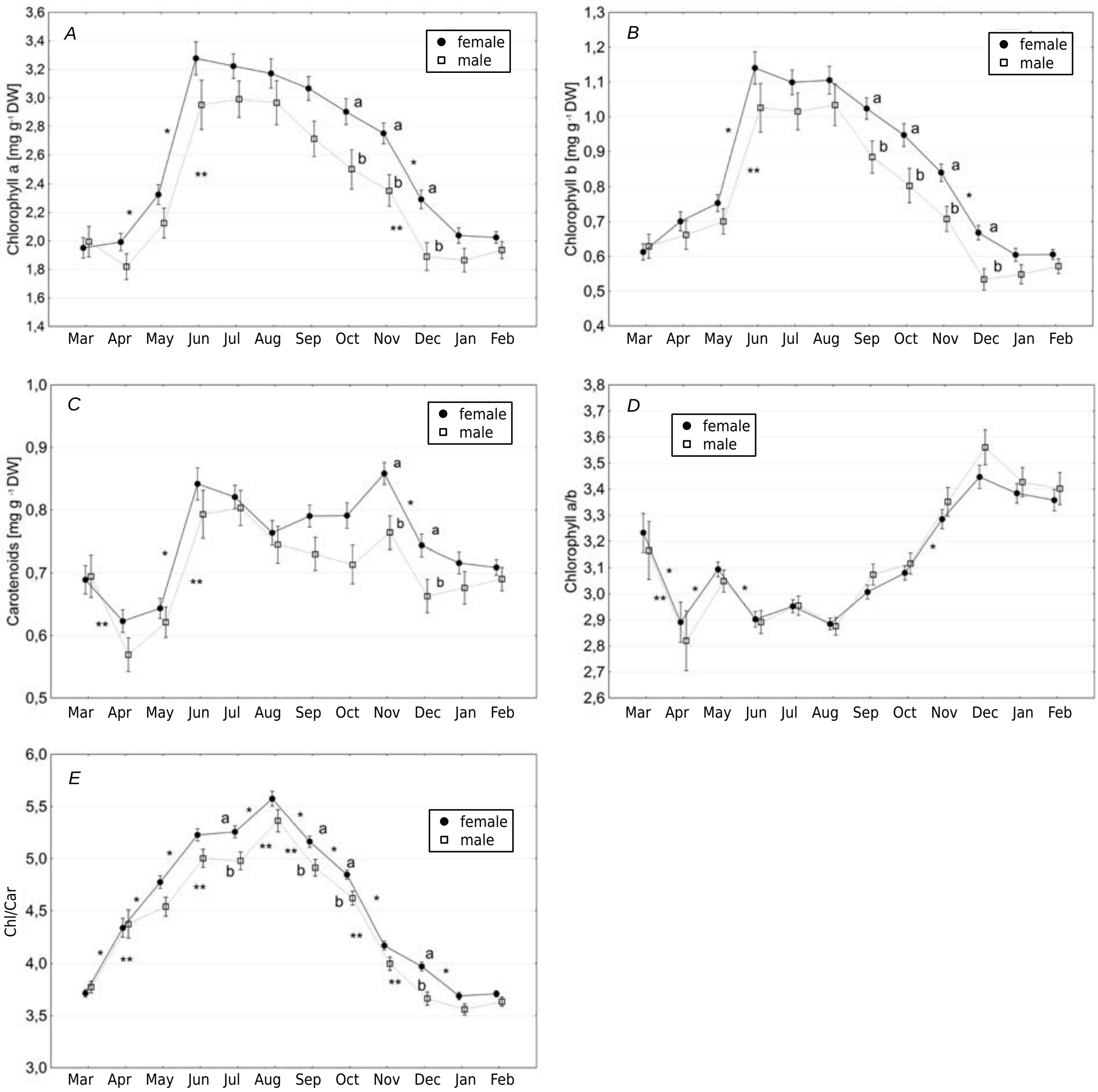

Fig. 3. Seasonal changes in the content of: (*A*) Chl *a*, (*B*) Chl *b*, (*C*) Car, (*D*) Chl *a/b* ratio and (*E*) Chl/Car ratio in extracts from the needles of male and female individuals of *Taxus baccata*. The means with the same letters are not significantly different (P > 0.05) within a given sampling dates. The months without letters are not significantly different between sexes. Each point is the mean of 24 measurements (±SE) for male and 48 measurements (±SE) for female. Asterisks (single * for female and double ** for male) indicates a significant difference between means of adjacent sampling dates (P < 0.05)

could be observed. They related to the content of Chl *a* (Fig. 3A), Chl *b* (Fig. 3B), and Chl/Car ratio (Fig. 3E). Other parameters: Chl *a/b* ratio (Fig. 3D), Car (Fig. 3C), PPIX (Fig. 4A), MgPPIX (Fig. 4B), Pchlide (Fig. 4C), Chlide *a* (Fig. 4D), and Chlide *b* (Fig. 4E) showed no significant differences between sexes. For all the studied parameters, except for Chl/Car ratio, interaction between sampling dates and sex was not statistically significant. Higher concentrations of Chl *a*, Chl *b*, and Car compounds were observed in female individuals; these differences were statistically significant in the autumn and winter period (Chl *a* from October to December, Chl *b* from September to December, Car from November to December).

Chl *a* level (Fig. 3A) in female individuals increased slightly from March (1.95 mg g^{-1} DW) to April (1.99 mg g^{-1} DW) and then intensively till June when it reached its maximum value (3.28 mg g^{-1} DW). From that moment onwards, it was slowly decreasing until November (2.75 mg g^{-1} DW) with a sudden decrease ending in January (2.03 mg g^{-1} DW) was observed. In male individuals, a slight decrease in the value of Chl *a* (from 1.99 mg g^{-1} DW in March to 1.82 mg g^{-1} DW in April) was initially observed and the increase in concentrations from April to May was slightly slower

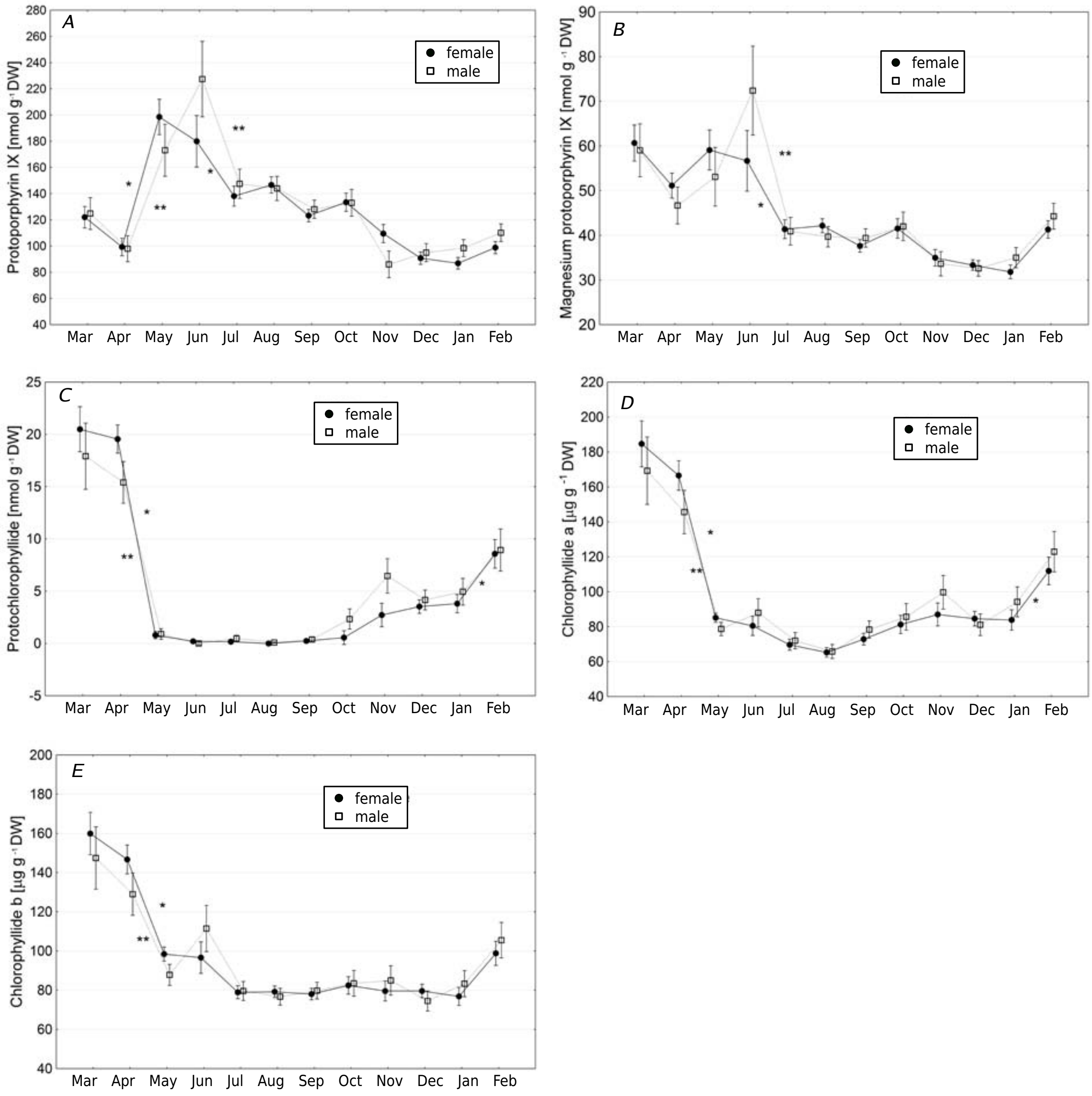

Fig. 4. Seasonal changes in the content of some chlorophyll intermediants and derivatives: (*A*) PPIX, (*B*) MgPPIX, (*C*) Pchlide, (*D*) Chlide *a*, and (*E*) Chlide *b*, in extracts from the needles of male and female individuals of *Taxus baccata*. Each point is the mean of 24 measurements (±SE) for male and 48 measurements (±SE) for female. Asterisks (single * for female and double ** for male) indicates a significant difference between means of adjacent sampling dates ($P < 0.05$)

in comparison to female individuals and still was not statistically significant. From May to June, an intense production of Chl *a* was already observed, although maximum is reached only in July (2.99 mg g^{-1} DW). High concentrations were established in the period from June to August followed by a fairly rapid decline of this value, ending in December (1.89 mg g^{-1} DW), therefore earlier than in female individuals.

A similar pattern was observed for Chl *b* (Fig. 3B). The level of this pigment was gradually increasing from March (0.61 mg g^{-1} DW for females and 0.63 mg g^{-1} DW for males) until May (0.75 mg g^{-1} DW for females and 0.70 mg g^{-1} DW for males), followed by a sudden increase to the level of 1.14 mg g^{-1} DW for females and 1.02 mg g^{-1} DW for males, reported in June. The high level was maintained for about 3 months and then from August, steady decline was reported to a level of 0.53 mg g^{-1} DW for males in December and a little later for females (0.60 mg g^{-1} DW in January).

Similar course of changes was observed in terms of Car (Fig. 3C) content from March to August, while slightly lower concentrations were observed in male individuals. Initially, the content of Car significantly decreased from March (0.69 mg g^{-1} DW) to April (0.62 mg g^{-1} DW for females and 0.57 mg g^{-1} DW for males), followed by a sudden increase in May (0.64 mg g^{-1} DW for females and 0.62 mg g^{-1} DW for males) till June (0.84 mg g^{-1} DW and 0.79 mg g^{-1} DW, respectively). Starting from August, in female individuals gradual increase of Car concentration was observed, which ends in November (0.86 mg g^{-1} DW), followed by an initially fast and subsequent slow decline in the content ending in February (0.71 mg g^{-1} DW). At the same time, the concentration of Car in male individuals decreases slightly from August to October, while in November it increases to a level of 0.76 mg g^{-1} DW followed by a rapid decrease to the value of 0.66 mg g^{-1} DW in December.

The change in the course of Chl *a/b* ratio curve (Fig. 3D) was similar in both sexes and reached its minimum in April (2.89 for females and 2.82 for males), while the maximum value was obtained in December, when it was slightly higher in male individuals (3.56 for males and 3.45 for females). In terms of Chl *a/b* ratio in the annual course, clear fluctuations were observed in the values from March to June and then there was a steady increase from August to December.

The value of Chl/Car ratio (Fig. 3E) in turn increased regardless of the sex in March and April. In the following months, in female individuals, there was a steady increase in the value with the slight slowdown in July and culmination in August established to the level of 5.57. In male individuals, from April to May and then from June to July, growth inhibition was observed followed by the occurrence of maximum similarity as in female individuals in August, and it was slightly lower (5.36). From that moment, an intense decline in the value of Chl/Car ratio in both sexes was observed lasting until January.

Early precursors of chlorophyll (PPIX and MgPPIX) reach the highest values in the period between May and June (Figs. 4A,B), then initially their levels decrease rapidly followed by a slow decrease until the initial level. Despite the lack of significant differences between male and female individuals in terms of the content of PPIX and MgPPIX, a shift in the concentration maximum for the analyzed compounds was observed, which for females occurs in May (198.5 nmol g^{-1} DW and 59.1 nmol g^{-1} DW, respectively) and for males in June (227.4 nmol g^{-1} DW and 72.4 nmol g^{-1} DW, respectively).

Different levels of concentrations are observed for more direct chlorophyll precursors: Pchlide, Chlide *a*, and Chlide *b* (Figs. 4C, D, E). The highest values are reached in March and April, being slightly higher in females in comparison to males. Then, their level decreases rapidly, starting to increase slowly in the autumn and winter period.

Age of needles differences

Significant differences between needles of different ages were observed only in the case of Chl *b* (Fig. 5B), MgPPIX (Fig. 6B), Pchlide (Fig. 6C), Chlide *b* (Fig. 6E) content, and Chl *a/b* ratio (Fig. 5D), and these differences were always caused by the needles classified as current-year needles. Considering only the annuals from the 1st to 3rd year (excluding the current increment), none of the investigated parameters showed statistical significance. Although in needles, a slight increase in the content of Chl *a* (Fig. 5A), Chl *b* (Fig. 5B), Car (Fig. 5C) and Chl/Car ratio (Fig. 5E) with age could be observed, and simultaneous decrease in the concentration of Pchlide (Fig. 6C), Chlide *a* (Fig. 6D), Chlide *b* (Fig. 6E), and Chl *a/b* ratio (Fig. 5D) with age was also reported.

In terms of Chl *a/b* ratio (Fig. 5D), high similarity in the course of curves for 1–3 annuals and different course for 0 annuals was observed, especially in the period from May to June, when the value for 0 annuals increases with simultaneous decrease reported in others. In the subsequent seasons, plots already have a similar course, while at the same time much higher value for 0 annuals is depicted. At the initial stage, current-year needles also showed high levels of concentrations of intermediates (PPIX and MgPPIX in May and Pchlide, Chlide *a*, and Chlide *b* in June) (Figs. 6A,B,C,D,E), which then suddenly decreased, and then in the subsequent part of the year showed much greater instability compared to 1–3 annuals.

Discussion

Differences observed by our group in terms of the occurrence of assimilation pigments (higher concentrations in female and lower in male individuals) are consistent with other studies conducted on dioecious plants. Lower content of assimilation pigments in male individuals was observed, i.e. in *Piper betle* L. (Kumar et al., 2006), *Populus deltoides* (Yang et al., 2011), *Ilex aquifolium* (Obeso et al., 1998), and *Ginkgo biloba* (Mori et al., 2000). Similarly, the leaf photosynthetic rates in *Acer negundo* (Dawson & Ehleringer, 1993) and *Salix arctica* (Dawson & Bliss, 1993) was higher in female individuals. On the contrary, in previous studies, Mitchell (1998) observed that male and female individuals from *T. brevifolia* and *T. baccata* growing under similar light conditions did not differ significantly among themselves.

Different results in relation to those obtained by our group may be caused by sampling dates because we observed in our study that the occurrence of differences increases or decreases depending on the month of the year. Differences in the content of assimilation pigments and the rate of photosynthesis also depend on the species. Unlike the European yew, leaves of male individuals in *Salix* and *Populus* contained more chlorophyll in comparison to female individuals (Bourdeau, 1958), while for *Populus trem-*

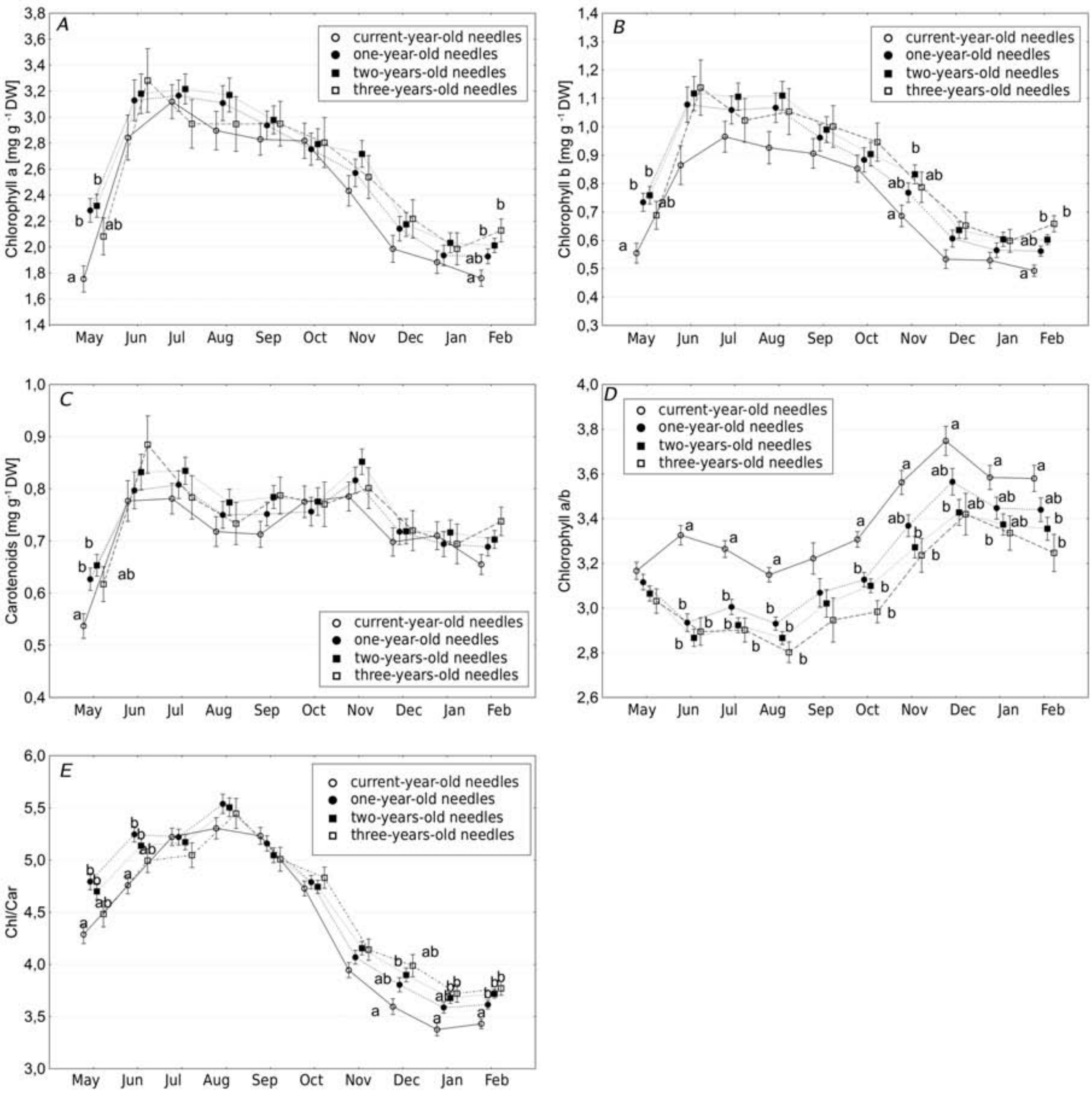

Fig. 5. Seasonal changes in the content of: (*A*) Chl *a*, (*B*) Chl *b*, (*C*) Car, (*D*) Chl *a/b* ratio and (*E*) Chl/Car ratio in extracts from the different age needles of *Taxus baccata*. The means with the same letters are not significantly different ($P > 0.05$) within a given sampling dates. The months without letters are not significantly different between age needles. Each point is the mean of 18 measurements (±SE)

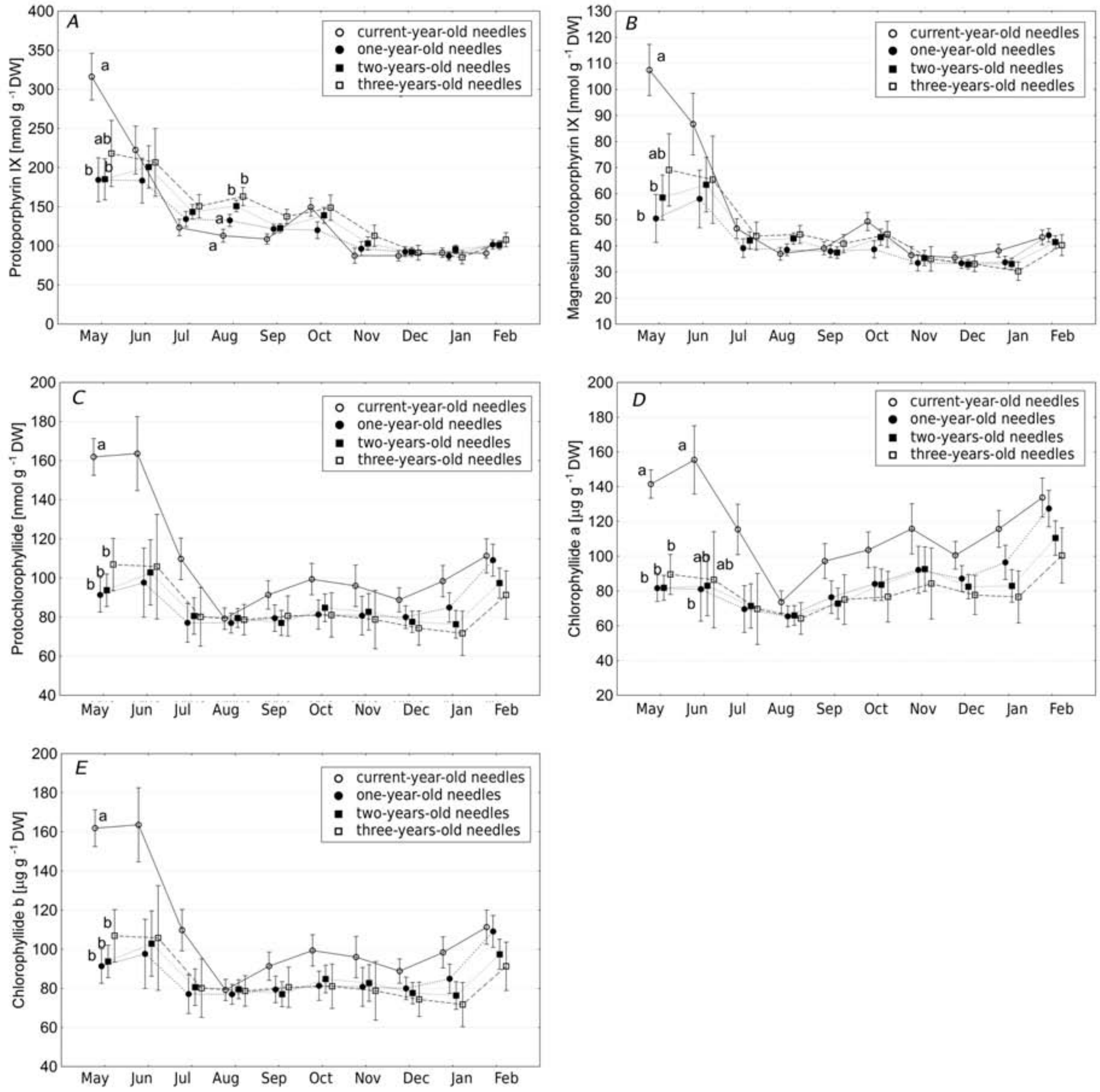

Fig. 6. Seasonal changes in the content of some chlorophyll intermediants and derivatives:(*A*) PPIX, (*B*) MgPPIX, (*C*) Pchlide, (*D*) Chlide *a*, and (*E*) Chlide *b* in extracts from the different age needles of *Taxus baccata*. The means with the same letters are not significantly different ($P > 0.05$) within a given sampling dates. The months without letters are not significantly different between age needles. Each point is the mean of 18 measurements (±SE)

uloides (Bourdeau, 1958) and *Silene latifolia* (Gehring & Monson, 1994), no difference in the rate of photosynthesis was observed.

Differences in the content of assimilation pigments could result from different reproductive effort born by both sexes. On the one hand, the main effort borne by male individuals is associated with the need to produce as much pollen as possible, and with vegetative growth, which provides increased distribution and greater pollen coverage (Obeso, 2002; Montesinos et al., 2006; Cedro & Iszkuło, 2011). On the other hand, female individuals bear much higher costs related to the production of seeds and fruits during the year, which occurs from June, i.e., since the end of intensive vegetative growth. The effort borne by female individuals is not only higher, but also more evenly distributed over the year. It begins in the spring with intensive vegetative growth and is further replaced by the effort associated with the production and maturation of fruits and seeds.

To meet this challenge, female individuals not only maintain higher level of assimilation pigments almost throughout the year, but also begin their production (especially Chl *a*) a little earlier in the spring and retain it longer in autumn. The need for earlier initiation of intensive spring growth in female individuals aimed at compensating a greater effort borne throughout the year may simultaneously cause an increased risk of late spring frost, over time leading to (as one of the factors) a change in the demographic structure in favor of male individuals in populations, which was, i.e., observed by Iszkuło et al. (2009).

Changes in the content of assimilation pigments observed in our study throughout the year have a similar pattern to that observed by other authors in deciduous trees and among trees such as *Fagus sylvatica* or *Morus alba* (García-Plazaola & Becerril, 2001; Sývacý & Sokmen, 2004) as well as coniferous trees, e.g., *T. cuspidata* (Swanberg & Verhoewen, 2002), *Picea abies*, and *Abies sibirica* (Silkina & Vinokurova, 2009). An increase observed in the amount of assimilation pigments in spring season is the result of chlorophyll biosynthesis prevalence over degradation processes. With the advent of summer, gradual increase in the prevalence of photodegradation processes of chlorophyll is observed, which is no longer compensated by biosynthesis, leading to a gradual decrease in the concentration of chlorophyll.

Because the process of production and decay of chlorophyll is controlled by light, it is important to maintain similar light conditions as possible during the sample collection. With high intensity of light, degradation processes outweigh the synthesis processes so that in plants growing in full sun, less chlorophyll content in comparison to plants growing in the shade was observed (García-Plazaola & Becerril, 2001; Swanberg & Verhoewen, 2002). In the shade, the content of Chl *b*, which allows for the absorption of light in a wider wavelength range, was observed. Therefore, Chl *a/b* ratio is often used as an indicator of plants' response to shading (Goncalves et al., 2001). Similar course of changes in Chl *a/b* ratio to that observed by our group in *T. baccata* was also present in *Piper betle* L. (Kumar et al., 2006) for which it reached the highest value in November (that is a month earlier than *T. baccata* observed by our group), and these values were slightly higher for male in comparison to female individuals. Higher value of Chl *a/b* ratio in male individuals may be caused by a higher degree of conversion of Chl *b* into Chl *a* by specific enzymes (Folly & Engel, 1999; Scheumann et al., 1999) or higher membrane sensitivity (Zu et al., 2010).

Two peaks of Car concentration observed by our group are probably related to the intensive spring production of assimilation pigments (first peak) and to a period of intensive chlorophyll degradation, which leads to an increased photooxidation risks (second peak). Simultaneously, differences in Car content occurring in the vicinity of the second maximum between male and female individuals may be associated with maturation and seed dispersal occurring at that time. The lower content of Car in male individuals was also observed in *Piper betle* L. (Kumar et al., 2006), *Populus deltoides* (Yang et al., 2011), and *Ginkgo biloba* (Mori et al., 2000). Female individuals exhibit higher levels of Car throughout the year so that they seem to be protected better against photooxidative damages (Gonçalves et al., 2001; Robakowski & Wyka, 2009).

Maximum content of early chlorophyll precursors (PPIX and MgPPIX) delayed by a month in male individuals is associated with later initiation of chlorophyll production and may indicate their delayed spring start, which at the same time constitutes a protection against late frost. Delayed start in the spring and earlier degradation of pigments in autumn may result from natural selection of male individuals under the influence of low temperatures. More sensitivity of male individuals against low temperatures in other species is also highlighted by other researchers (Mori et al., 2000; Kumar et al., 2006; Yang et al., 2011).

Because of assimilation pigments synthesis is highly affected by light conditions it is generally difficult to divide the effect of light from the needles' age. In the case of studies conducted by our group, samples were collected from the outer part of the crown, which along with morphological comb-like structure of the needles' arrangement on the shoots in *T. baccata* should minimize the influence of self-shading effect. Comparing the content of assimilation pigments in needles of different age tested, it can be observed that in terms of Chl *a*, this content does not depend on age throughout the year, while in Chl *b* significantly lower concentrations throughout the year can be found in the youngest needles. In turn, Jach and Ceulemans (2000) observed higher concentration of chlorophyll in this year's needles of *Pinus sylvestris* in comparison to last year's, i.e., in June and September. In the first year, we observed a gradual increase in the content of chlorophyll, while during the second year, a decrease in the concentration of chlorophyll was reported. Differences observed by our group in *T. baccata* in relation to *Pinus sylvestris* may be caused by, i.e., lifetime of the needles in particular species, which in terms of European yew are estimated at 2–6 and even eight years as reported by other authors (Szaniawski, 1978). According to Szaniawski (1978), the ability of European yew needles to perform photosynthesis decreases with age and drops to 50% in seven-year-old needles compared to young needles.

Differences observed by our group in terms of Car content between different year-old needles exhibit a slight increase in concentrations, which probably results from their gradual accumulation along with needles' age. Increased instability in three-year-old needles throughout the year may indicate early symptoms of aging. The value of Chl/Car ratio also slightly increases with needles' age, which is caused by a faster increase in the content of chlorophyll and a slightly lower increase in Car content. Increase in Car content along with age of needles was also observed in, i.e., Scots pine (Miazek & Ledakowicz, 2013). The value of Chl/Car ratio was lower in younger tissues of Scots pine's and also increased with age. For young, susceptible tissues, higher content of Car in relation to chlorophyll can serve as a protective function against oxidative stress or excessive radiation. During vegetative season, chlorophyll is decomposed earlier than Car, which leads to a decrease in the Chl/Car ratio. In studies conducted by our group, we did not observe any decrease in this value with needles' age, which may indicate that three-year-old needles have not initialized the process of aging on a large scale.

High value of Chl *a/b* ratio observed in young *T. baccata* needles reported by our group is mainly caused by a relatively low content of Chl *b*. In turn, decreasing Chl *a/b* ratio in progressively older *T. baccata* needles is similar to changes observed in *Cupressus sempervirens* aging needles (La Porta et al., 2006). In case of *Cupressus sempervirens*, authors explained the changes by decreasing amount of Chl *a* resulting from aging processes of needles. In our case, decrease in Chl *a/b* ratio was mainly caused by the increased content of Chl *b* in relation to Chl *a*. Decreased values of this coefficient observed by our group in one-, two-, and three-year-old *T. baccata* needles in relation to this year's needles may indicate better acclimatization of older needles' year to changing light conditions related to, i.e., increased self-shading from year to year.

Moreover, in terms of Scots pine (Miazek & Ledakowicz, 2013), individuals exposed to intense light were characterized by higher Chl *a/b* ratio in comparison to those growing under less intense light. According to Zu et al. (2010), increase in Chl *a/b* ratio may be caused by the increasing degree of decomposition of grana containing photosystem PSII.

The youngest needles during the most intense growth phase exhibit very high concentration of early chlorophyll precursors (PPIX and MgPPIX), which is most likely related to the need of rapid production of the appropriate level of pigments ensuring an adequate level of assimilation. More direct precursors such as Pchlide, Chlide *a*, and Chlide *b* reach their maxima in the youngest needles a month later in relation to early precursors, which is caused by the increased production of assimilation pigments. In subsequent three years, the level of all precursors in needles remains very balanced with no signs of decline with increased age. Our studies do not indicate the existence of significant differences in the content of assimilation pigments and their precursors associated with the process of needles' aging during the first four years, but only small differences related to their development in the first year of life.

Conclusions

Differences in the content of assimilation pigments can constitute a kind of biochemical marker of sex under the maintenance of specific sampling conditions. The most suitable period of plant material harvesting ranges from November to December, when the greatest differences in the concentrations of both Chl *a*, Chl *b* and Car as well are observed.

Analyzing the impact of needles' age on the concentrations of compounds tested, it can be concluded that the youngest needles (current-year-old needles) are most different from others (both in terms of concentration range and dynamics of seasonal changes). In turn, among three-year-old needles, seasonal fluctuations slowly start to increase, which may constitute first symptoms of aging. When collecting biological material for research purposes, it is best to choose one-year-old or two-year-old needles, which, on the one hand, are already fully developed and stable and, on the other hand, do not demonstrate any symptoms of aging.

In addition, one should also take into account a great impact of lighting conditions on the concentrations of the compounds described. To obtain meaningful results, the influence of light intensity should be excluded as much as possible, which is often difficult to achieve in field conditions; however, it may allow for the identification of sex in case of artificial cultivation of propagation material carried out under controlled conditions. The studies conducted also indicate a quite high variability of the traits studied throughout the year, depending on the sex of individuals investigated and age of needles that indicates the necessity of a strict consideration of these parameters when comparing the results obtained. The difference observed between male and female individuals need to be confirmed by further analysis of biochemical compounds, and in the future, it may become an element of biochemical markers' system that identifies sex.

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