Identification of European orchids by determination of the anthocyanin concentration during development of the blossoms[1]

W. Uphoff

Institut für anwendungsbezogene Forschung, Fachhochschule Kiel, Legienstr. 35, D-2300 Kiel (Federal Republic of Germany), 2 October 1981

Summary. The blossoms of many species of European orchids contain a conspicuous blue cyanidinglycoside (Orchicyanin), which has bathochromic characteristics. This makes it possible to observe the formation of this anthocyanin during the development of the blossoms spectrophotometrically. The concentrations of Orchicyanin vary in a way that is typical of the species and genera.

In an earlier publication I described a complex cyanidin-glucoside, of a conspicuous blue to violet color, which is characteristic of the sub-family Orchidoideae, and which was named Orchicyanin I, due to its occurrence and its chemical structure[2]. This anthocyanin contains a co-pigment causing a bathochromic effect, i.e. the light absorption shifts towards the long wave range leading to a change in color from red to a bluish-violet. Similar effects in the Commelinaceae have been reported by Golo et al.[3], Hoshino et al.[4], and Stirton and Harborne[5]. Of all the anthocyanins identified in European orchids, Orchicyanin I alone has these marked bathochromic properties. Therefore its biosynthesis can be recognized by the shift of the absorption maximum of the blossom-extract towards the long wave range. The rate of synthesis of the Orchicyanin is dependent on the species of orchid, as well as on the stage of development of the blossoms. For this reason we have systematically examined the European orchids in order to ascertain reactions typical of the species and genera and if possible to establish a method of identifying them down to the smallest possible taxonomic degree.

Experimental. As the inflorescence is arranged spicately in most European orchids, blossoms of all stages of development are available on a single plant. Before the blossom samples were gathered, the length of the inflorescence, as well as the lengths of the sections bearing blossoms and buds were ascertained. The numbers of blossoms and buds were counted. Then we measured the distance and the number of blossoms between the blossoms that were to be sampled and the boundary between buds and open blossoms. The blossoms and buds of the partially-opened inflorescence were taken in fixed intervals on a vertical line from the top to the base of the inflorescence. They were then put into vials and processed as soon as possible.

In the 1st stage of the analysis, the blossom pigments were separated by the usual method of TLC[6,7] on cellulose with n-butanol-acetic acid-water (4:1:5). Then they were eluted in 0.9 M acetic acid and their concentration and absorption maxima were determined. As ascertained in previous experiments 0.9 M acetic acid was especially suitable for this purpose. This is due to its capacities as a solvent, the chemical stability of the anthocyanins, and the marked bathochromic characteristics that Orchicyanin I shows in this solvent. In the further progress of the studies the absorption spectra of the blossom-extracts without previous separation were established. For this purpose one blossom or bud, excluding anthers and ovary, was weighed and then triturated with an exactly measured volume of 0.9 M acetic acid. The mixture was then centrifuged. The top layer, consisting of a clear anthocyanin solution, was separated from the colorless residue with the help of a pipette. Then its spectrum of absorption in the visible range was measured.

Results. Repeated determination of the absorption spectra of the pure anthocyanins which were isolated from *Dactylorhiza maculata, D. majalis* and *Orchis mascula,* with 0.9 M acetic acid as a solvent, gave the following absorption maxima:

Orchicyanin I: 538, 350, 265 nm

Orchicyanin II: 513, 273 nm

Therefore the bathochromic shift of Orchicyanin I, in the visible range important for our measurements, is 25 nm. The absorption maximum of cyanidin 3,5-diglucoside (511 nm) is close to that of Orchicyanin II, and is furthermore to be found only in small concentrations in the orchid blossoms studied. For this reason, its influence on the absorption maxima of the blossom-extracts is negligible.

Dactylorhiza maculata. Figure 1 shows graphically the concentration of Orchicyanin I relative to the total concentration of anthocyanins as determined in the experiment. It also shows the corresponding absorption maxima of the blossom-extracts as dependent on the stage of development of *Dactylorhiza maculata*'s blossoms. A direct correlation between the concentration of Orchicyanin I and the absorption maximum is to be seen.

Figure 2 shows a further curve, which is derived from measurements of the absorption maxima only of *Dactylorhiza maculata* from different plants of the same origin. Figure 3 shows the results from samples of different

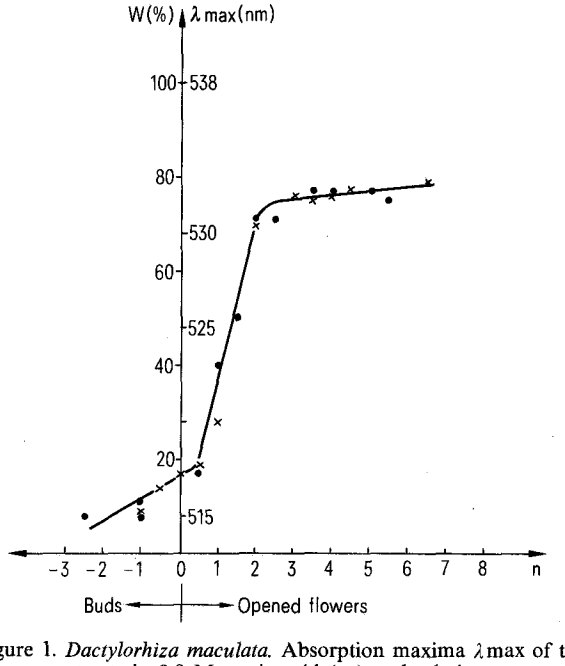


Figure 1. *Dactylorhiza maculata.* Absorption maxima λmax of the blossom-extracts in 0.9 M acetic acid (×) and relative concentrations referring to cyanin (●), pigment contents referred to the sum of all anthocyanins. n, Number of open blossoms (n positive) resp. buds (n negative) to the boundary line buds/open blossoms. One opened blossom corresponds to about 1.5 days.

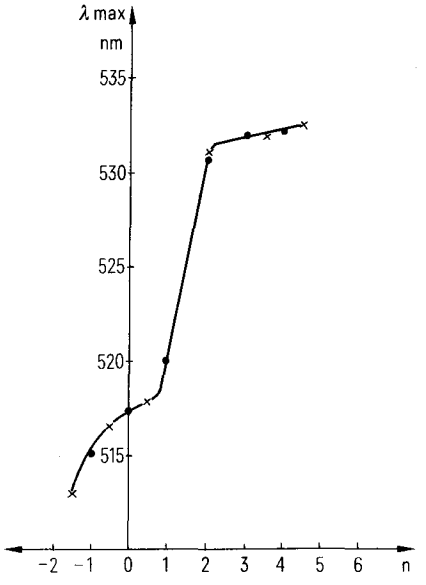
geographical origin (North Germany, the Alps, and the Mediterranean area). Although all the curves show a certain breadth of variation, they all follow the same course with 3 phases of development; the buds contain small concentrations of Orchicyanin I (λmax < 520 nm), there is a sharp increase in Orchicyanin I production in the opening blossoms and during the 1st 1-2 days of anthesis, and a high level of concentration of this anthocyanin thereafter until the blossom withers.

Dactylorhiza majalis (fig. 4). This species, like *D. maculata*, shows a 3 phase development with an increase in Orchicyanin I production when the blossoms open. It is clearly distinguished from this species, however, by the markedly smaller increase in the slope of the curve, so that the Orchicyanin I concentration in the fully developed blossom is lower then in *D. maculata*. Also remarkable in this species is the large spectrum of variation. Plants with intensively colored blossoms contain a higher percentage of Orchicyanin I throughout all phases of development than do light-coloured specimens.

Orchis mascula (fig. 5) shows a completely different reaction in comparison to the species of *Dactylorhiza* studied so far. This species already synthesizes mainly Orchicyanin I in an early stage of development of the buds. This high level is constant during the entire time of blossoming. It is notable that even relatively small deviations in the Orchicyanin concentration in all the plants stand out very clearly. Characteristic of *Orchis mascula* is a flat but marked

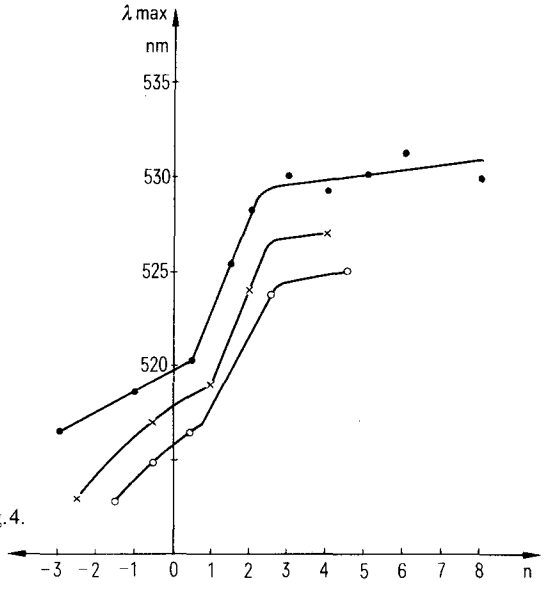
Figures 2-8. Absorption maxima of the blossom-extracts of European orchids. Also see explanations under figure 1.

Fig. 2.



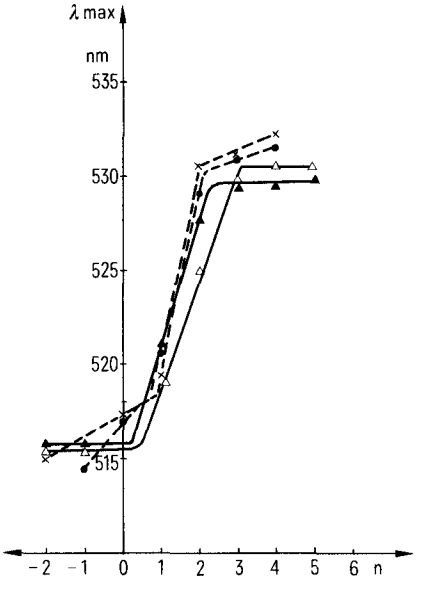
Dactylorhiza maculata ssp. *fuchsii*. ●, × Different plants from the same habitat.

Fig. 4.



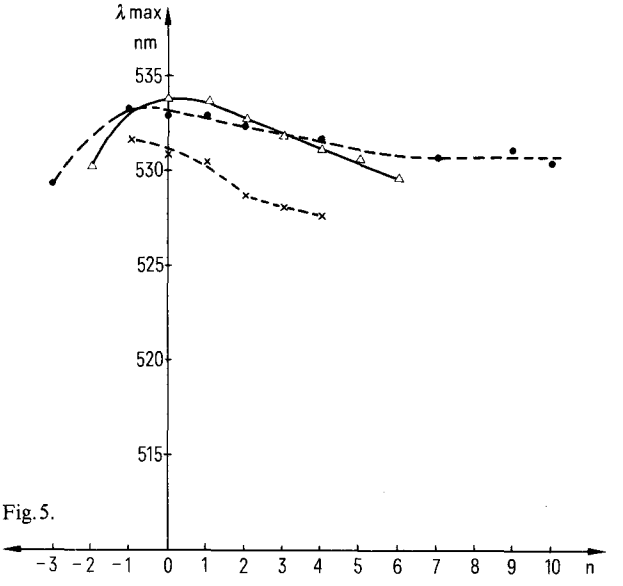
Dactylorhiza majalis. ● Northern Germany, deep colored flowers; ○ Northern Germany; × Bavarian Alps.

Fig. 3.



Dactylorhiza maculata s.l. ● Etrurian Apennines (*D. fuchsii*?); × Central Alps (*D. fuchsii*); △ Liguria (*D. maculata* s.str.); ▲ North Germany (*D. maculata* s.str.).

Fig. 5.



Orchis mascula. ● Bavarian Alps; △ Etrurian Apennines; × Southern Alps (ssp. *signifera*).

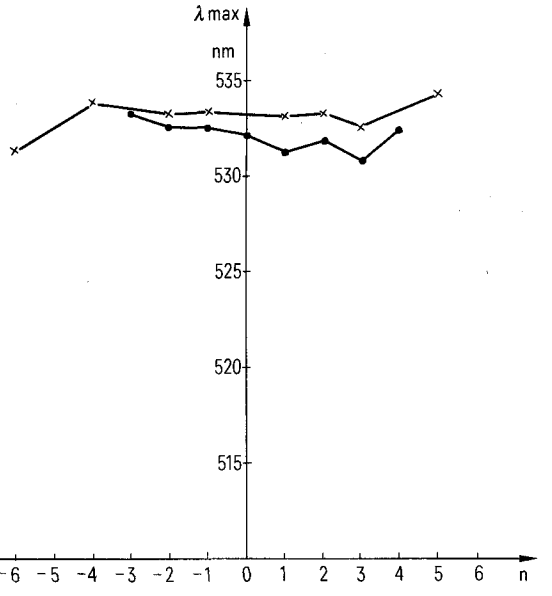


Fig. 6.

Orchis morio ssp. *morio.* ● North Germany (Isle of Fehmarn); × Tuscany, near Florence.

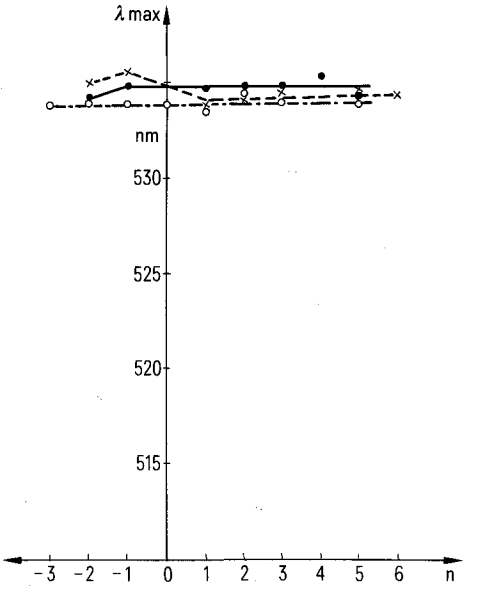


Fig. 8.

Gymnadenia conopsea. ● Alps, 2000 m; × Alps, 800 m.

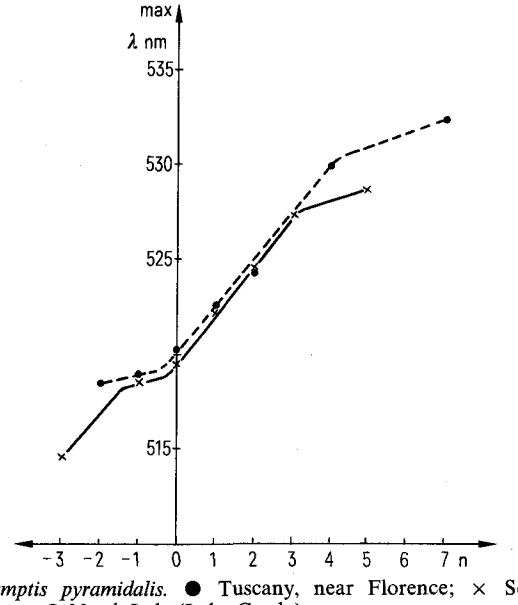


Fig. 7.

Anacamptis pyramidalis. ● Tuscany, near Florence; × South Germany; ○ North Italy (Lake Garda).

maximum at the commencement of anthesis. The subspecies signifera shows a similar course; however, the Orchicyanin I concentration is somewhat lower and its maximum comes clearly before the commencement of anthesis in this relatively light-coloured species.

Orchis morio (fig. 6) is similar to *Orchis mascula* with its high λ max-values over the entire range and its corresponding high relative concentrations of Orchicyanin I. The curve maximum at the commencement of anthesis, which is typical of *Orchis mascula,* is, however, missing.

Anacamptis pyramidalis (fig. 7) is distinguished by values of absorption maxima, and therewith concentrations of Orchicyanin I, that are especially high and almost constant throughout all phases of development. This finding confirms the relationship of the species *Anacamptis* and *Orchis.*

Gymnadenia conopsea (fig. 8) is similar to the *Dactylorhiza* species. A relatively slow, but almost constant increase of the curve throughout all stages of development of the blossom is typical. Mountain-forms contain more Orchicyanin I, especially at the end of blossoming time, than do specimens that grow in lower regions.

Gymnadenia odoratissima has not been studied much so far. It could be determined, however, that this species is similar to *G. conopsea.* The curves obtained from the blossoms at a late stage of development showed a more marked increase at the commencement of anthesis than was found for *G. conopsea.*

Discussion. All in all, it can be stated that the absorption maxima, and therefore the relative concentrations of Orchicyanin I in the blossom-extracts show a typical course during the development of the orchid blossoms, and that these have a taxonomic validity. The occurence of Orchicyanin is characteristic of the sub-family Orchidoideae. The fundamental course of the curve representing the relative concentrations of Orchicyanin is typical for the genus, and its microstructure is characteristic of the species. Further studies will show whether a further taxonomic differentiation is possible, by introducing the individual-concentration, i.e. the concentration of Orchicyanin in relation to the weight of the unprocessed individual blossom. Preliminary experiments have given positive results.

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