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Acclimation Changes of Flavonoids in Needles of Conifers during Heat and Drought Stress 2015

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Abstract: The long-term harsh climate conditions in 2015 distorted already from June up to November in all study trees of *Tsuga* and *Taxus* the intracellular organization of the needles. Intimately involved in these repressive processes were the flavanols, a small subgroup of the flavonoids. They were not only deposited in vacuoles of conifer needles but also in the nuclei and chromosomes. Among the many flavonoids the small group of catechin derivatives and polymers named flavanols can exclusively be stained blue with DMACA (dimethylaminocinnamaldehyde). From mid-July onward, the vacuolar flavanols of the epidermal cell layers were gradually diminished as evidenced by decreasing blue staining of nuclei and vacuoles. Subsequently, in August also the large spongy mesophyll cells showed the flavanols decreasing progressively. Apparently, the antioxidant flavanols operate as oxygen radical scavengers. (ROS) were used up during the harsh environmental stress conditions. Both, *Tsuga* and *Taxus* reacted in this way. However, it is quite surprising that in all study trees the palisade cells did not contain such vacuolar flavanols. Only these cells were in June the first to show a loss of chlorophyll from chloroplasts as well as an efflux of flavanols from the nuclei. Conversely, from September onward another group of phenols, the yellow-staining flavanols were newly formed in the palisade cells and later on also in the mesophyll cells. Obviously, they were assembled finally to stabilize finally the fragile cell sites. Summing up, the present study shows by cytological studies that the climatic conditions in 2015 produced the worst disturbance of subcellular structures observed since 2000 when our studies on nuclear phenols in needles of conifers were initiated.

Keywords: climate impact; needle; conifer; chloroplast; flavonoids; flavanol

1. Introduction

In nuclei of conifers the flavanols (flavane-3-ols) can be bound to the basic histone proteins [1]. Histones with associated flavanols turn blue with DMACA reagent and the intensity of the color depends on the amounts of flavanols [2]. It is very important that this unexpected finding based on histology was confirmed by use of pure physical methods [3].

A widely accepted competent role of flavanols is the pronounced property to eliminate or scavenge toxic oxygen species as free radicals [4]. Such a mechanism would provide a high degree of protection especially for the nuclei with its DNA and protein molecules being particular targets for damaging superoxide radicals [5].

Solar radiation within a tree canopy changes from very direct sunlight to scattered diffuse and shady which results in variable physiological processes and photomorphogenetic events. By microscopy of the histochemical reactions the stress symptoms can be detected very early before macroscopic judgement [6].

Thus far, in this study, dealing with the monthly proceeding stress of conifers caused by heat and drought in 2015, the flavanols started by mid-summer to decline. The upper epidermis was the first tissue showing a gradual loss of the dark blue staining vacuolar flavanols. This is surely triggered by its direct exposition to the harsh light radiation whereas the subjacent tissues, the palisade cells and the spongy mesophyll underlie more scattered light conditions. Nevertheless, the loss of flavanols towards November was apparent.

In broad-leaved plant species the chloroplasts of the mesophyll tissues are readily protected against UV-radiation by yellow compounds from the flavonol group [7,8]. For example, quercetin derivatives as wide-spread flavonols can be bound to proteins, so triggering significant antioxidant effects against oxygen-induced cytotoxicity [9,10].

In the foregoing years, for example 2003 and 2006, the nuclear flavanols of conifers were shown to disappear for a few days if exposed to a short-period of drought and heat [11]. Now, in 2015, the climate change culminated in rather long hazardous periods of UV-radiation and drought.

Particularly evident was the decrease of chlorophyll resulting in a change of photosynthetic plastids into etioplasts. Ultimately from September through November, the increased synthesis of yellow colored flavonoids was a strategy to stop a definitive cellular disintegration. These flavonoid pigments, based on quercetin and kaempferol derivatives, operated apparently as survival molecules. The evolutionarily rather distant genotypes of *Taxus* and *Tsuga* species showed anyway a largely similar behavior anyway in the phenol-based response mechanisms to severe stress.

2. Materials and Methods

2.1. Climate Conditions of 2015

Europe experienced 2015 as the warmest year since 1880 when weather-stations were established. This points out that the critical temperatures were measured in the shade as usual but also in sun-exposed sites of the canopy. This was necessary considering that during the hottest days in July and August the over-heated needles were exposed for some hours to solar radiation. Supra-optimal temperatures of 50 to 55 °C occurred in full sun during afternoon, when in the shade, between 30 to 33 °C was registered. So far, the difference between directly sun-exposed and shaded positions was about 20 °C.

The longest rainless period lasted three weeks in August and the maximal temperatures reached just during this time at 12 days up to 32 °C (Table 1) or 52 °C in full sun. Under such Mediterranean climate conditions the study trees surely had serious problems of adaptation.

Table 1. Monthly climate data from 2015 indicating average temperatures (aT), max. Temperatures. (max. T, number of days), average rainfall (aR, mm), and max rainfall, (max R, number of days).

	aT °C	Max T °C	aR mm	Max R mm
March	10	1 × 17	20	1 × 8
April	15	3 × 22	49	1 × 24
May	19	2 × 27	102	2 × 24
June	21	3 × 29	38	2 × 21
July	27	7 × 34	20	2 × 5
August	24	12 × 32	44	1 × 21
September	19	1 × 28	33	1 × 8
October	11	2 × 21	61	3 × 13
November	11	15 × 17	57	1 × 53
December	8	3 × 12	15	1 × 5

The exact climate data were obtained from the weather station Weihenstephan of the University Munich which is situated at about 4 km away from the experimental field. The total precipitation in 2015 was only 472 mm. There was an exceptional period in May with 102 mm of rainfall. The soil moisture conditions during the following 4 months (June to September) were less than half of the normal values.

2.2. Plant Material

The study trees were *Tsuga canadensis* L. (hemlock), *Tsuga heterophylla* (western hemlock L.), as well as the two varieties *Taxus baccata* L. (yew) and *Taxus repens*. From each genotype 2 trees were investigated. The crown of each tree was at least partially exposed to sun. *Tsuga repens*, as a typical understory tree, grew under limited light conditions. Sampling was performed from early May to late December in 2015. The "warm" January 2016 was additionally included because of unexpected reorganization of the needles. The trees were randomly located in the Botanical Garden of the University Munich. The needles were exclusively sampled from current year shoots.

2.3. Histology

Blue staining of flavanols was carried out for 20 min with DMACA (1% p-dimethylamino-cinnamaldehyde in 1.5 N methanolic sulfuric acid). The study of nuclei and plastids was performed with freehand sections of fresh needles immediately before microscopy.

Longisections were made from the epidermal layers about 3 mm in length. Transverse sections about 70 to 150 µm thick were made to investigate the palisade and mesophyll cells. For one microscopic study about 100 to 120 needle sections from 5 needles per study tree were examined. About 70% of the needles were taken from the south half of the crown. Sampling was performed weekly or in 10-day intervals. After extreme hot days extra sampling was performed one or two days later.

In *Tsuga can.* the blue colored flavanols catechin, epicatechin, epigallocatechin and their dimeric molecules B1, B2 including their condensed proanthocyanidins were determined by DMACA [12] using HPLC techniques [13]. These flavanols were also found in *T. baccata* [11].

Not all flavonoids are generally yellow. Yellow groups of flavonoids (myricetins and quercetins) were identified in anthers or needles of *T. baccata* [14]. In an extensive study by Krauze-Baranowska a rich chemical diversity of flavonoids in the numerous cultivars from *T. baccata* was found; to name a few, quercetins, kaempferols, rutinoside myricetin, or rutinoside quercetin [15].

The use of the natural reagent DPBA (diphenylboric acid-ß-aminoethyl ester) yielded more intense yellow colors for flavonoids whereby kaempferol tended more strongly towards yellow than quercetin.

The HPLC-post-column identification of flavones and flavonols was achieved by comparison with authentic standards such as quercetin, myricetin, apigenin, kaempferol and luteolin [16]. However, it is impossible to separate a mixture of different flavonoids located in a mesophyll vacuole by HPLC. For DNA staining 4, 6 diamidino-2-phenylindole (DAPI) was used. The DNA was excitated at 365 nm and fluoresced in the blue spectral area. This reagent proved to be simple and fast for the tissues of *Taxus* and *Tsuga*. Propidium iodide gave a red color for DNA using UV-395-440 or was visualized with a long pass 585 nm emission filter. The presence of green chlorophyll was visualized directly in the light microscope or by the bright red microscopic fluorescence.

Microphotographs were obtained with a Zeiss light microscope and a fluorescence microscope. Filter sets for propidium iodide were G 395-440, FT 460. For DAPI G436, FT510, LP 520 were used (the same filter can be applied for propidium iodide). Light microscopic observations were measured by the computer system Fujitsu-Siemens-Nicon.

3. Results

3.1. Loss of the Optimal Nuclear Assembly from May to August

In May when growth is initiated, typical cell lineages were formed with a structural clonal character. The identical size, color and exact circular shape of the red stained DNAs is characteristic of a functional lineage (Figure 1a). The blue color of the adjacent 2 lineages with 4 cells each due to flavanols which contribute by their antioxidant reactions to the stability of both proteins' chromosomes. As a first sign of severe developmental irregularity, there was a lineage with 3 rather diffuse blue patches whereas the fourth nucleus was compacted but not correctly rounded (*). In the second lineage (**), four telophase nuclei could be seen with a slightly variable flavanol staining. There was a somewhat different lateral tearing stress. The brown burnt lineage nuclei sampled by June from a sun-exposed needle (*T. bacc*.) exhibited a clear sign of total damage (***).

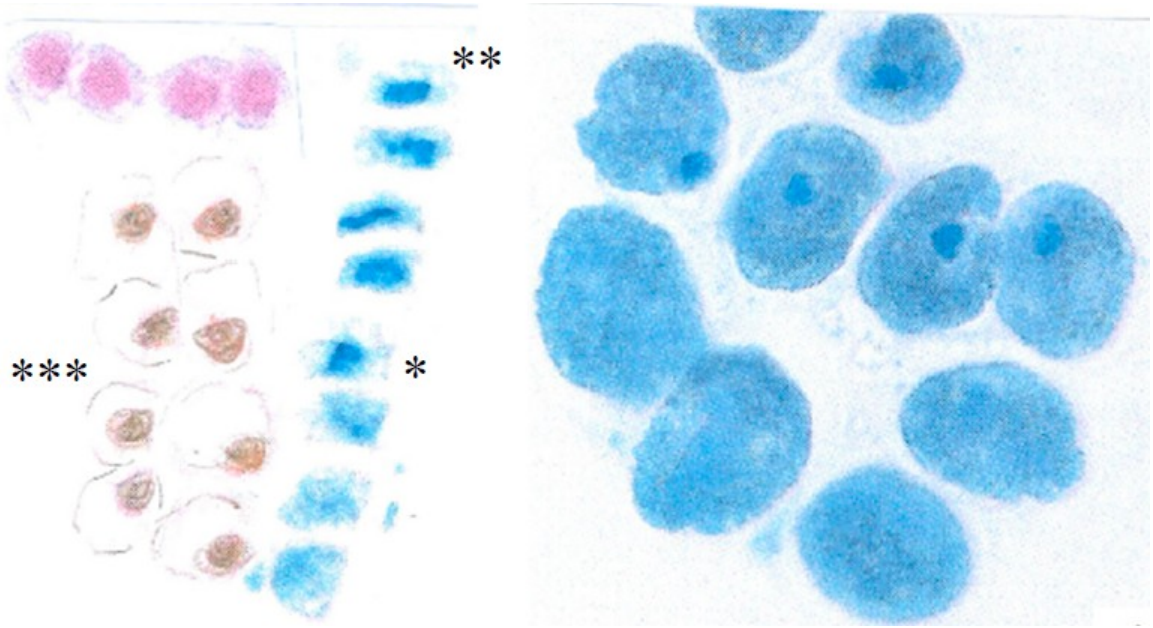


Figure 1. Eight brown burned lineage nuclei and 4 healthy nuclei stained with propidium iodide for DNA (**a**) and expanding lineage cells (**b**) from T. baccata, blue colors are DMACA treated.

In *Tax. rep.*, a lineage with 4 expanding cells developed by late June spherical mesophyll cells (Figure 1b). All nuclei were only 3 to 4 µm in diameter. In fact, the prevailing majority of active nuclei from conifers ranged between 7 and 9 µm in diameter, as evidenced by all previous investigations since 2000. A number of factors could account for this undersized development. In any case, such a small nucleus is severely silenced. In the 4 lowermost cells, the blue staining of nuclei was blotted out.

The study trees of *Taxus* and *Tsuga* were examined on the loss of nuclear viability (Figure 2). This criterion was checked by the degree of the non-circular shape of the nuclei. The recently formed nuclei were in May to a large extent ball-shaped. However, later on up to late August they turned to irregular and multiangular forms. This would coincide with a series of dislocated binding targets and the too distant sites of interactions.

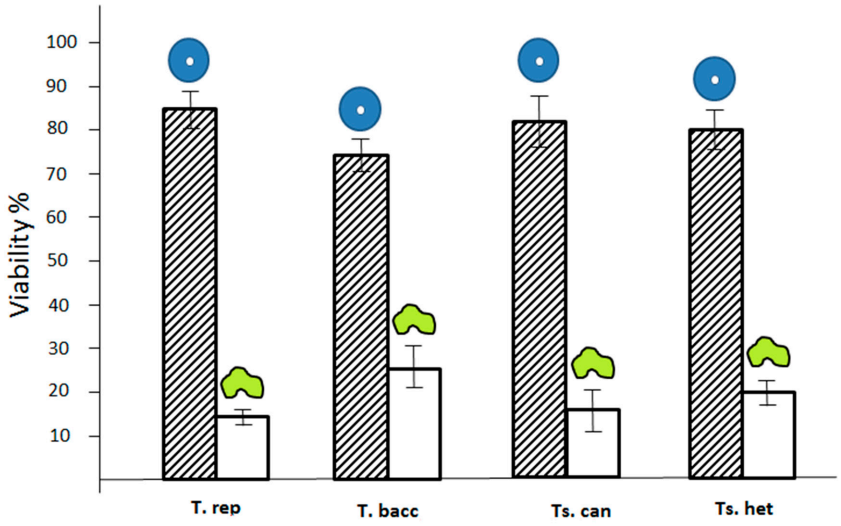
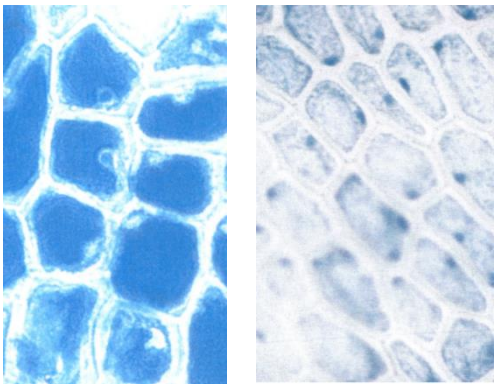


Figure 2. Loss of nuclear viability = high viability = low viability with T. rep (Taxus repens), T. bacc (Taxus baccata), Ts. can (Tsuga cannadensis) and Ts. het (Tsuga heterophilla).

3.2. Early Breakdown of the Epidermal Flavanol Barrier against UV-Radiation

The cell wall of the upper epidermis is physically very stable, about 10 µm thick and contains yellow compounds of the flavonol type. The epidermal vacuoles themselves were 15 to 20 µm in height and showed a rather dense flavanol accumulation up to late-July (Figure 3a).



(**a**) (**b**)

Figure 3. Longitudinal section of the upper epidermis (**a**) and lower epidermis (**b**), both DMACA treated.

By mid-August there began a visible downregulation of the dense flavanols which occupied practically the entire upper epidermal cell layers (Figure 3a). Some nuclei had already lost the flavanols as clearly evidenced by five white spots. In two cells the nucleus was surrounded by a white ring, possibly indicating that the endoplasmic reticulum attached to the nuclear membrane.

Each cell contained only one large vacuole filled in each case with the blue phenolic molecules (Figure 3a). The disturbance of the protective radiation shield continued progressively up to September and October. The reduction of flavanols revealed that the needles were unable to maintain an effective anti-radiation shield. This situation might be explained by the autumnal decrease in light and heat which needs no further presence of flavanols.

As a rule, in all study trees the lower epidermis did not develop such a strong flavanol shield as the upper one (Figure 3b). This is best explained by the lower light incidence. In addition, less light stress may help to maintain the optimal conditions of nuclear vitality, namely flavanol binding.

3.3. Early Breakdown in July of the Young Seed Wings from Tsuga

Both *Tsuga species* produced structurally similar cells throughout the oval-sized wing of the seed wings. Their development began in 2015 in mid-June, about the time when the young cones were 6 mm in length. However, under the stress conditions in 2015 from early July onwards nearly all nuclei from the seed wings lost their globular shape (Figure 4). Instead, they attained gradually an irregular, flattened form.

Then, during the last days of July, four extreme heat periods combined with drought were disastrous for the seed wings. Exactly 33 °C in the shade and 55 °C in full sun were measured. As a consequence, instead of the blue stained cell walls and nuclei a number of seed wings turned to pigmented tan-brown (Figure 5b). Three days after this heat stress the flavanols of many nuclei were clearly seen to leak out. About 80% of the nuclei had lost much of their normal, globular shape, which resulted in thin stripes strongly pressed towards the cell walls. A correct globular nucleus (* upper corner, left) was found in fore-going years with normal climate conditions). In 2015 the deformation progressed rapidly so that in September the needles of *Tsuga can*. lost nearly 100% of the nuclear flavanols.

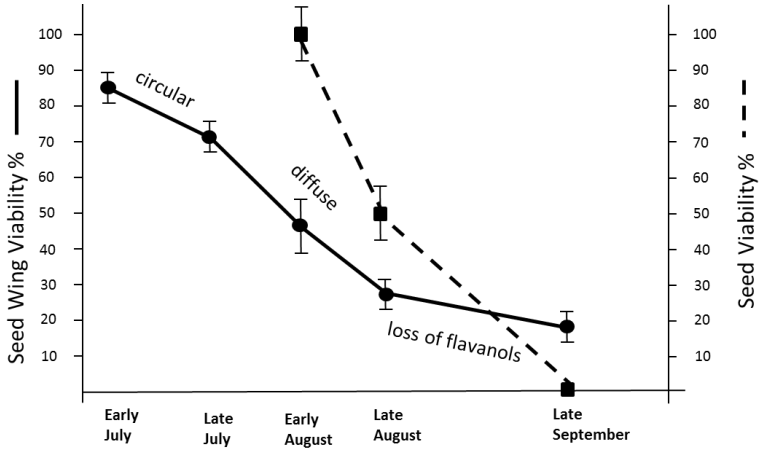
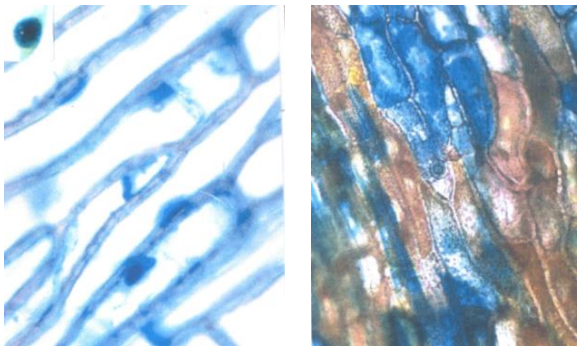


Figure 4. Loss of the globular shape of the nuclei from seed wings ● and loss of seed viability ■.



(**a**) (**b**)

Figure 5. Normal staining of healthy seed wings (**a**) and brown burned seed wings (**b**); blue colors are DMACA treated, cells from a and b have the same size.

The seeds located at the bottom of the wings started to develop in August. However the nuclei appeared rather diffuse and most of them even were from beginning to dry. Compared to *Tsuga can.*, the species *Tsuga het.* was at first somewhat more stable in nuclear flavanols, but then broadly lost them in late September. Even brown, dry sectors of the seed wings could be observed.

3.4. Loss of the Flavanols Promotes the Onset of Intracellular Structural Mismatch

The decline of the needles was quite variable in the different needle tissues. The progressing deterioration was characterized by a reduction of flavanols. An overview is presented in Table 2 to give in brief a guide for the focal points of the following investigations.

Table 2. Start and monthly sequence of the decline of the 4 needle tissues (Columns 1 and 2). Furthermore, the velocity and intensity of the decline of the four tissues and genotypes (Columns 3 and 4) are shown.

Start of Decline Tissue 1	Start of Decline Month 2	Velocity of Decline Tissue 3	Intensity of Decline Genotype 4
1. Upper epidermis	early July	medium	*Tsu can.* strong
2. Palisade cells	late July	very high	*Tsu het.* medium
3. Mesophyll cells	late August	high	*Tax back.* medium
4. Lower epidermis	late August	medium	*Tax rep.* low

3.5. First Signs of Breakdown of the Epidermal Flavanols by Mid-July

The cell wall of the upper epidermis is physically very stable, about 10 µm thick and contains yellow compounds of the flavonol type. The epidermal vacuoles themselves were 15–20 µm in height and showed a rather dense flavanol accumulation up to late-June (Figure 3a). However, towards mid-July, the first signs of decrease in vacuolar flavanols could be observed (middle sector of Figure 3a). Then, some blue staining nuclei could be seen in the fragmentary blue epidermis (Figure 3a).

From late August to early September there was a progressive loss of vacuolar flavanols and their epidermal shielding function. All trees from *Tsuga* (Figure 3b) and *Taxus* followed this pattern. As a rule, the lower epidermis stained generally somewhat less blue than the upper one (not shown).

3.6. Very Early Decrease of Green Chloroplasts of the Palisade Cells in Late July

The change from green chloroplasts to etioplasts from May 2015 to January 2016 is demonstrated for *Taxus* and *Tsuga* in Figure 6; the vertical bars are the SE; $n = 50$.

As already pointed out, direct excessive sunlight combined with drought is critical for a needle. Regarding the photosynthetic palisade cells the direct hazardous effect of severe sunlight is surely attenuated by the overlying dense flavanol-barrier of the upper epidermis. This shelter of security might be approached by the palisade cells which were in 2015 had no vacuolar flavanols in both *Taxus* and *Tsuga*. In the foregoing years, both the size and number of such vacuoles were limited and more or less variable. However, already in June and more so in July there were 10 days with extreme heat stress near 30 or up to 34 °C. This coincided in the palisades with an early onset of chlorophyll degradation in July and an accumulation of yellow flavonoids in vacuoles. In *Tax. bacc.*, the palisade cells, if stained on flavanols, showed only small remnants of blue, probably traces of diffused nuclei (Figure 6a). As a so far unique arrangement in *Tsuga can.*, the chlorophyll leached completely away from the chloroplasts and remained with its green color attached to the lower cell walls (Figure 6b).

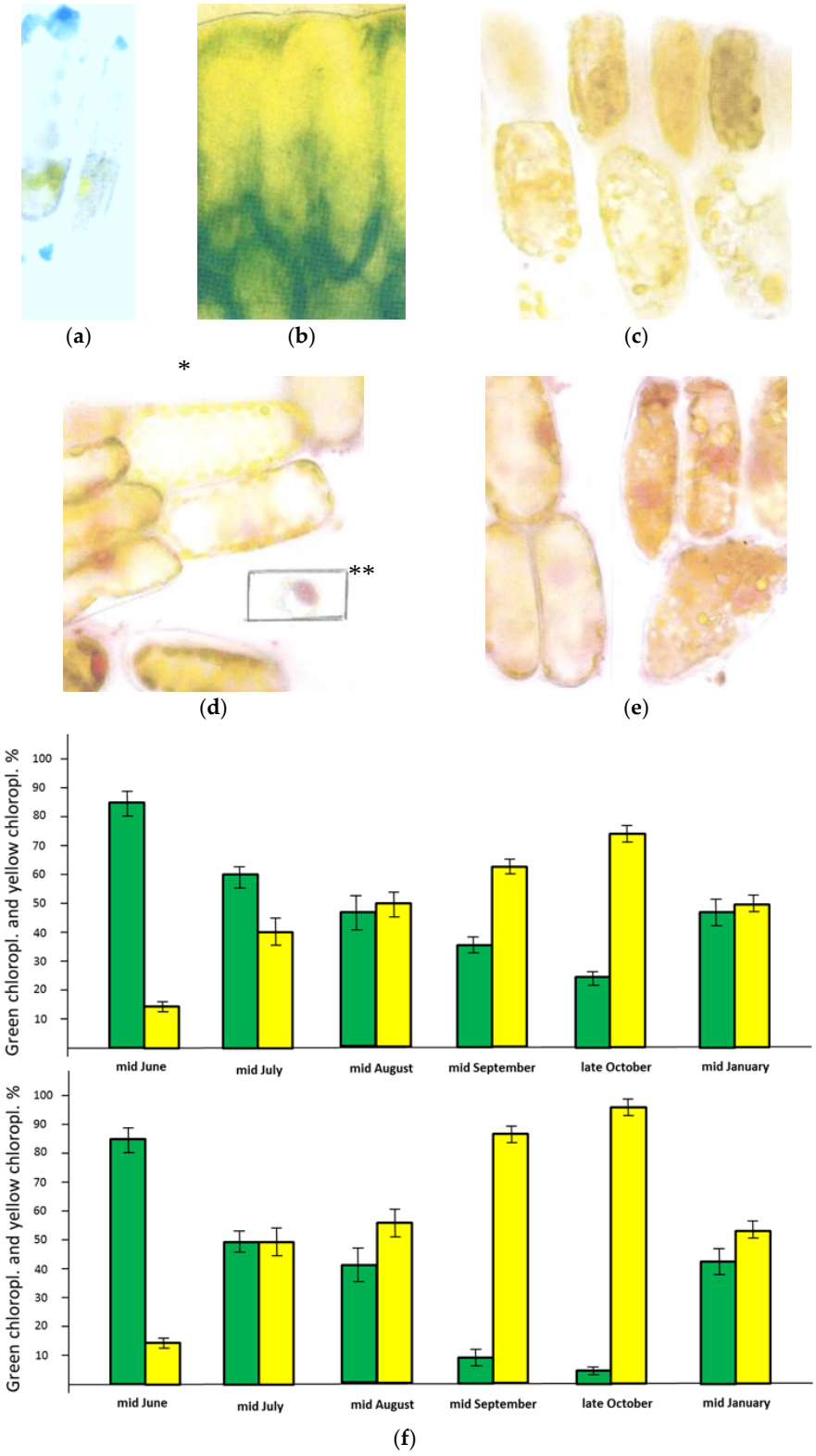


Figure 6. (**a**–**f**) Distribution of flavanols (blue), flavonols (yellow), and DNA (red) in the cells of the palisade tissue.

In a further example of *Tsuga het.*, there were not even remnants of nuclear flavanols but the cells had very pronounced diffuse yellow flavanols (Figure 6c). In *Tax. bacc.* a rather typical flavanol-free example of diffused yellow cytoplasm was observed (Figure 6c).

Generally, all cells from the four study trees suffered from a very diffuse red stained DNA as shown in Figure 6d. As a control a small cell with a non-leaching nucleus sampled in 2012 is shown (Figure 6d*). Diffuse yellow cytoplasm mixed with a cloudy reddish tint was found in the cells are shown. In one cell, yellow plastids were seen along the cell wall (Figure 6d**).

In *Tsuga can*. (Figure 6e) the reddish staining DNA was more prominent and rather diffused. Some small yellow vacuoles were additionally seen.

The quantitative change of green and yellow chloroplasts is documented by the corresponding columns (Figure 6f). From May to October the monthly data were sampled. In mid-August the chloroplasts with a yellow tint surpassed those with still green colors. This tendency was by late October much more pronounced in *Tsu. can.* than in *Tax. bacc*.

3.7. Retarded Breakdown of the Flavanols from the Spongy Mesophyll in Late August

About four weeks after the palisade cells the spongy mesophyll cells of all study trees started with a decline of the flavanols by mid-August. Later on, up to November, the *Tsuga* trees were somewhat more affected than the *Taxus* group.

Generally, the spongy mesophyll tissue was characterized by large, partially undulated cells which were separated by extended intercellular air spaces (Figure 7). The large and small mesophyll cells behaved distinctly because the larger ones stained more intensely blue than the smaller ones. The latter lost the flavanols mostly earlier (Figure 7a).

A change from blue to greenish and yellow cytoplasm was obvious as a response to the climate stress in all study trees even in adjacent cells (Figure 7c). the first signs of flavanol degradation were also observed (Figure 7c).

Leaching of flavanols out from the cells was found in Euonymus japonica Thunb. (Figure 7d). The mode of antioxidant chlorophyll protection in mesophyll cells appears to be also valid for other plant genera. In the broad-leafed E. japonica, the mesophyll cells were fully packed with flavanols and they could be easily confused with those of *Taxus or Tsuga*. Noteworthy in this context, the nuclei of E. japonica had no flavanols.

The special characteristics of these mesophyll cells need some explanation. It is readily striking that especially the largest mesophyll cells were occupied totally by dark blue staining vacuolar flavanols, similar to the epidermis cells (Figure 7e). The cytoplasm between vacuole and cell wall was usually barely seen. However, just in these dark blue staining giant vacuoles there were quite a lot of chloroplasts together with one blue colored nucleus (white *). The chloroplasts could be only seen without any prior staining. Adjacent, an oval rather large nucleus is developed in a smaller nearly flavanol-free cell. The non-circular mesophyll cells yielded diameters with an average of roughly 50 to 80 µm. This cell type contained up to 60 to 100 chloroplasts. The smaller mesophyll cells were not so strongly overloaded with flavanols and exhibited about **30** to **50** chloroplasts.

Finally during September and later on, there appeared greenish mesophyll cells indicating a mixture of blue flavanols with yellow flavanols (Figure 7f). Finally, only the yellow flavanols were left over and the diffused DNA of the nuclei was leaching around in the cell (Figure 7g). This feature was valid for all study trees.

3.8. The Curious Restoration of Green Chloroplasts in January 2016

As an outstanding event, the synthesis of new flavanols and a regreening of etioplasts began in early January 2016 (Figure 8). From early January 2016 onward the colorless chloroplasts returned to greenish colors. The number of functional chloroplasts increased steadily and they reached up to mid-February green structures nearly similar to those in May. Only one large mesophyll cell was for the most part diffuse (Figure 8a).

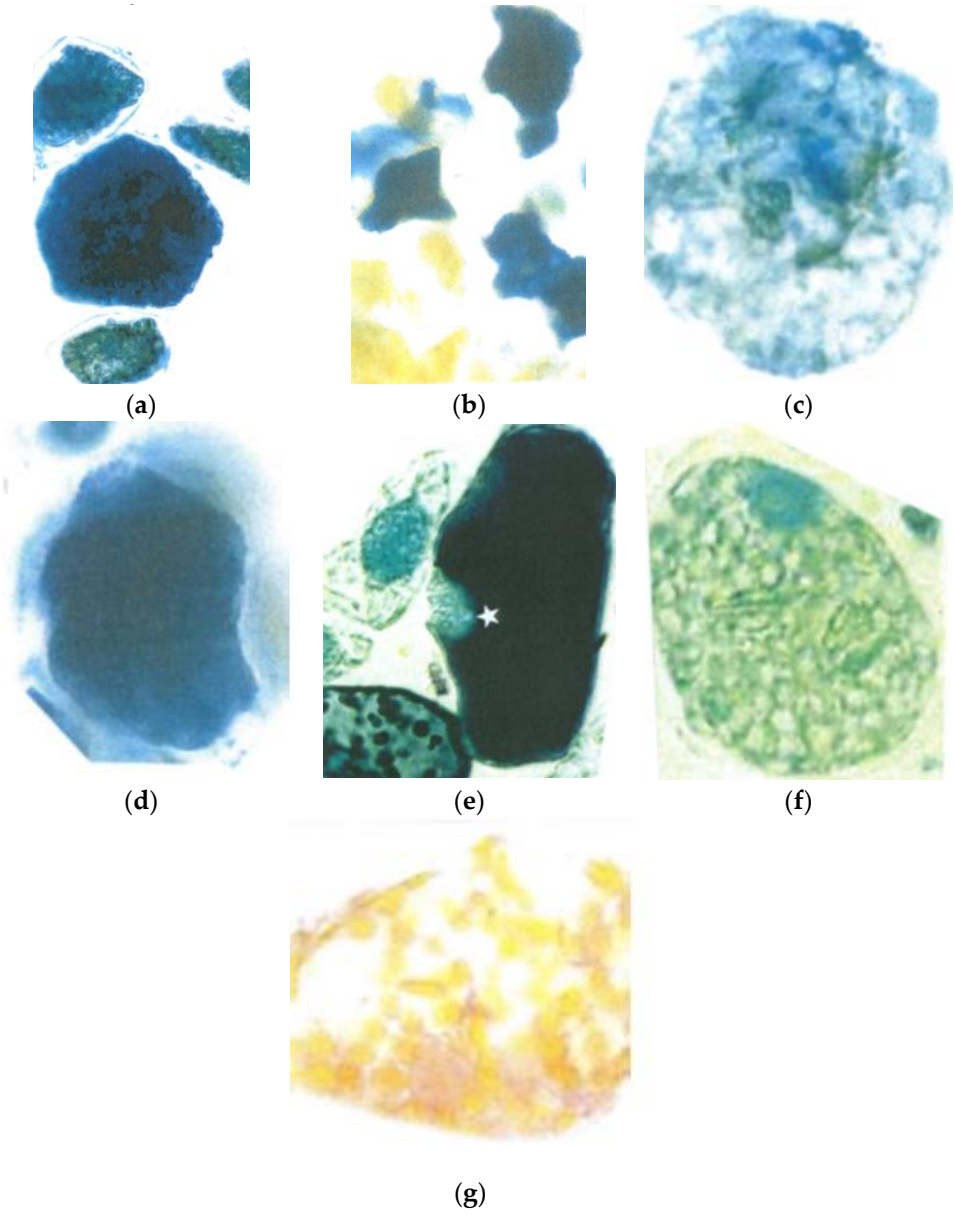


Figure 7. (**a**–**g**) Loss of the flavanols in the spongy mesophyll cells.

A few "wintry" days in mid-January with temperatures ranging from about minus 4 °C to plus 5 °C had no consequences on re-greening. From 20–30 January there was a period with elevated temperatures reaching in the early afternoon 5 to 13 °C. In *T. baccata* re-greened chloroplasts in the mesophyll cells were evident (Figure 8a). In *Taxus rep.*, green chloroplasts of the mesophyll cells were still dipped in a diffuse green solution (Figure 8b).

In both *Tsuga* species, the palisade cells showed green-yellow chloroplasts (Figure 8c). Those of *Tsu. het.* were more yellow (Figure 8d).

During the course of summer and harvest there was an increasing loss of chlorophyll accompanied by the synthesis of flavonols like quercetin and kaempferol derivates (Figure 9). Figure 9 is the "schematic representation of the entire study".

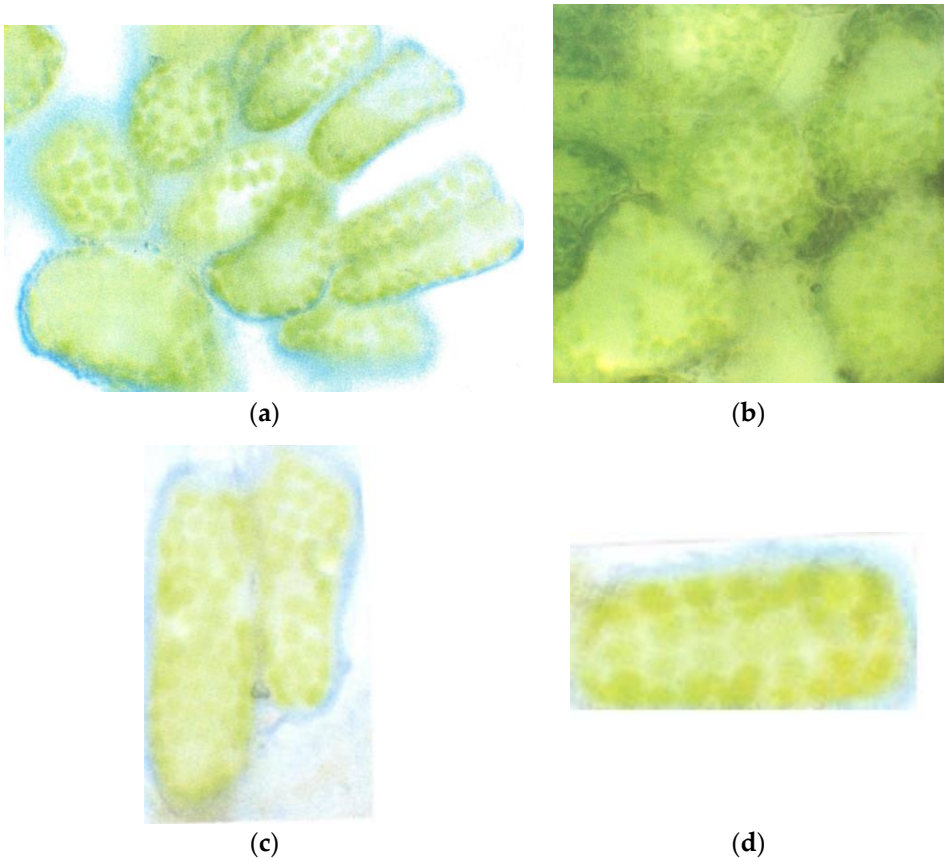


Figure 8. (**a**-**d**) Restoration of the chloroplasts during winter time.

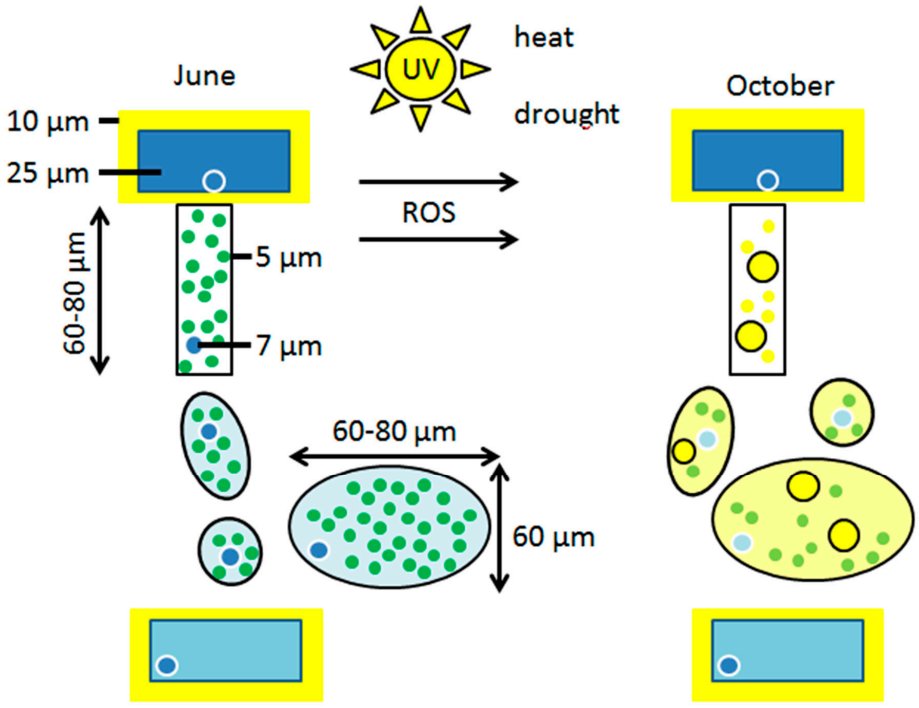


Figure 9. Schematic representation of the entire study.

4. Discussion

4.1. Early Beginning of Cellular Disorganization in June 2015

In 2013 and 2014, a few short heat periods affected for some days the nuclei insofar as they lost the flavanols. Perhaps as a consequence, the study trees did not store enough reserves and showed already in May–June 2015 the first signs of decreasing nuclear flavanols were observed. Catechin prevented paraquat-induced necrosis of strawberries [17]. In plants, extreme UV-radiation overwhelmed the scavenging activities of antioxidative compounds resulting in genomic sequestration [18,19]. The first signs of defective nuclear viability in mid-May 2015 in all study trees of *Taxus* and *Tsuga* apparently resulted later in summer in a promotion of the ageing processes. Hereby, a coincidental symptom was the diffusing of flavanols and DNA. In this context, it is particularly noteworthy that leaching of both electrolytes and organic compounds out of callus cells from cherry trees as caused by abscisic acid was inhibited by the flavanol catechin [20].

There is evidence that points to damaged nucleoporins at the nuclear surface which then lose the control of import and export [21]. This finding coincided with a weakening of nuclear membranes by drought damaged phospholipids of wheat [22]. Environmental factors, such as high temperatures above 32 °C were found to promote ageing as well as disordered membrane structures [23]. In fact, oxidative processes pave the way toward senescence and must be overcome by a spill over of reductive potentials [5].

In August the blue stained nuclei of the investigated conifer needles turned to a pale color. The conifer needles, although severely damaged, remained in 2015 alive up to the rest period. Hereby, the vacuolar flavanols were nearly completely used up as antioxidants to retard or inhibit the final disastrous radical-induced lethal effects. The very critical de-greening stage was encountered by an increased synthesis of yellow flavonols in both cytoplasm and chromoplasts. Obviously, the high chemical diversity of the so-called secondary phenolic molecules, allows the plants to establish an effective defense potential against the multiple climatic and biotic stress events [24].

4.2. Flavanols Guarantee an Antioxidative Protection of the Overstresses Mesophyll Chloroplasts

Viewed under evolutionary aspects, the conifer needles have adapted both their anatomy and molecular chemistry to survive severe threats of the environment. Therefore, the upper epidermis of all study trees is de facto no longer a compacted, continuous defense barrier with abundant antioxidant flavanols against light and UV interception. Their catechol structure with the B-ring provides the most active antioxidative power among flavonoids [4]. More generalized, stress survival against active ROS redox networks is reported to be achieved by molecule groups with a high reductive potential [25]. By developing a valid needle architecture and activation of corresponding transcription factors the plants can efficiently elevate the resistance against sunlight and UV radiation [26].

Beneath the epidermal shield the vertically very elongated palisade cells constitute a tissue without vacuolar flavanols. The reasons for this peculiar and perhaps risky absence of flavanols are difficult to ascertain but might be attributed to a much higher photosynthetic potential because of more space for chloroplasts.

The risk of a poor adaption of conifers under the conditions of global warming was emphasized by Voltas et al. [27]. It is not known to what degree the epidermis exerts its antioxidative umbrella effect downward into the palisade chloroplasts. In any case, in the unusually vertical strongly stretched palisade cells most chloroplasts have the advantage of being located more distant from the damaging impact of sunlight.

As the mesophyll cells are located even more distant from the upper sun-exposed epidermis than the palisade cells, one could conclude that they actually need lower amounts of protective flavanols. However by contrast, the mesophyll is as rich in flavanols as the upper epidermis. In order to find an explanation for this phenomenon, an efficient photosynthate production is linked with the crucial impact of dangerous ROS. A high level of toxic oxygen during photosynthesis might constitute a

destructive scenario [5], overall under the extreme climate conditions of 2015. The photosynthetic oxidation of water and the formation triplet oxygens is in any case a complicated machinery [28]. Up to 80 to100 chloroplasts exist in the large mesophyll cell of the conifers. In order to avoid the immense risk of a detrimental accumulation of ROS molecules, there must be an abundant presence of scavenging antioxidant flavanols. In addition, H_2O_2 produced from chloroplasts moves to the nucleus to intervene in gene expression [29].

The flavanols are capable to yielding a maximal antioxidative potential, if compared to flavonols and flavones [4]. Obviously, this is the case in the investigated conifers. In this way, the palisade cells, being without vacuolar flavanols, during environmental overstress like 2015 inevitably enter into a critical situation. Indeed, in all study trees the palisade chloroplasts started to lose the chlorophyll very early already in mid-July (2015). This was about three to four weeks earlier compared with the large spongy mesophyll cells. To reiterate, [30] postulated that polyphenols play an important role in genomic stability.

4.3. Flavanoid Deposition in Severely Stressed Needles

A fundamental somewhat provocative viewpoint should be anticipated. Increasing concentrations of yellow-colored quercetin derivates significantly inhibited the growth of young pea plants [31] as well as the proliferation of human cancer cells [32]. When in August 2015 needle growth declined, the mesophyll flavanols started to diminish, and then the accelerated ageing of the cells together with yellow flavonoids became more apparent. In addition the increase of kaempferols and quercetins under the influence of UV light was reported to stabilize the genome structures [18].

Flavonoids as signaling molecules have many functions related to growth regulation and defense processes [33]. The effect of drought stress on broccoli (*Brassica oleracea* L.) was the enforced appearance of yellow flavonoids [34].

In a similar way, antioxidant yellow colored flavonols accumulate in chloroplasts under high sunlight irradiance [7]. In keeping with this concept, especially the palisade and mesophyll cells of *Taxus* and *Tsuga* produced rather early such yellow flavonoids in the droughty year 2015.

Principally, photosynthesis is a critical event due to imbalances of the redox components setting up flavonoid synthesis as antioxidants.

The present investigations fully confirm earlier studies that flavanols have a high potential to eliminate even the extremely harsh effects of phytotoxic superoxide radicals induced by the application of paraquat (methyl viologen) [17]. Upon ageing, nucleoporins of the nuclear membranes lose their selective functionality [21]. Intact membranes in all trees of this study decayed very early in 2015. Especially, membrane destabilization combined with leaching in both nuclei and chloroplasts may be a primary point of needle senescence caused by climate stress already in July.

4.4. Restoration of the Chloroplasts in Mid-Winter 2015/2016

The needles of the study trees by the end of 2015 suffered from a severe deficiency of reserve compounds. It is to suggest that in January 2016 special signaling molecules in chloroplasts like flavonoids approached, rapidly the improved climate conditions for a renewal of normal metabolic conditions. Agati et al. emphasized the beneficial role of flavonoids in such situations [7].

Abiotic stress was found to induce adaptive adjustments for optimizing special physiological traits that successfully restore photosynthesis [35]. It is a real sensation that in the photosynthetic tissues the chlorophyll-free etioplasts were restored to green chloroplasts during January. Apparently, this event is absolutely unexplored by scientist. Thus, to the authors' knowledge no scientific data on this event are available in the literature. Indeed, the extreme and curious climate conditions in 2015 are unique. In addition, there was also a return of the protective epidermal flavanols, the antioxidant caretakers of the entire needles.

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