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A Cytogenetic Method for Examining the Vitality of Spruces

By

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With 10 Figures

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Summary

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This paper shows and describes in detail a cytogenetic method for examining the vitality of spruces and discusses its practical applicability. It is based on observation and critical examination of chromosome aberrations in the metaphase nuclei of root meristems. Not only the frequency of such damages but also the kind of aberrations lead to conclusions about the vitality of spruce population; moreover is it possible for the first time to examine the vitality of young spruces.

Zusammenfassung

MÜLLER M., GUTTENBERGER H., GRILL D., DRUŠKOVIČ B. & PARADIŽ J. 1991. Eine cytogenetische Methode zur Vitalitätsprüfung von Fichten. – Phyton (Horn, Austria) 31 (1): 143–155, mit 10 Abbildungen. – Englisch mit deutscher Zusammenfassung.

In der vorliegenden Arbeit wird eine cytogenetische Methode zur Vitalitätsprüfung von Fichten vorgestellt, ausführlich beschrieben und ihre Anwendbarkeit für die Praxis diskutiert. Sie beruht auf der Beobachtung und Beurteilung von Chromosomenaberrationen in Metaphase-Kernen der Wurzelmeristeme. Nicht nur die Häu-

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figkeit des Auftretens solcher Schäden, sondern auch die Art der Aberrationen lassen Schlüsse auf den Vitalitätszustand des Fichtenbestandes zu; erstmals ist es auch möglich, Vitalitätsprüfungen an jungen Fichten durchzuführen.

Introduction

Several natural and anthropogene influences have a large impact on the vitality of spruces. The traditional methods of bioindication (sulfur analysis of needles, judging of tree crown condition etc.) did not result in a definite judgement on vitality. Therefore we have tried to elaborate a method which adds up to methods used so far thus leading to a definite statement. The assumption that abnormalities within the genetic material of meristemes could give information about the state of vitality is the centre of our considerations.

Experiences with eukaryontic cells in other investigations showed that chemical influences resp. radiation lead to chromosome aberrations (e. g. WOLFF 1961, GOUD 1967, FUJIWARA & KONDO 1972, BENDER & al. 1974, TOMKINS & GRANT 1976, JAYABALAN & RAO 1987, BRIAND & KAPOOR 1989). It must be mentioned, however, that in these investigations the chemicals resp. the radiation was conveyed to the tissues directly and in high doses.

Here, however, we are concerned with the influx of chemicals of minor concentration but longer duration of influence in correlation with natural symptoms which seems to complicate the situation. But after the basic study of DRUŠKOVIČ 1988a there are definite clues that the method of „Cytogenetic Bioindication" will bring about results which lead to possible conclusions about the vitality of spruces. It is the aim of this paper to modify, complete, and standardize the suggested method thus presenting it for its practical use.

Material and Methods

Plants and locations of experiment, primary treatment and fixation

The spruce (*Picea abies* (L.) Karsten) serves as bioindicator tree. Normally wild grown spruces are planted in clay pots at the locations in spring. The trees should be 3 to 5 years old, the soil is taken from the location. Three trees per location are planted and watered, each spruce is surrounded by a screen-wire with a netting of 0.5–1 cm. The spruces should be exposed at the edge of a forest. The root tips of these trees are taken out in autumn and spring. The spruces can stay in the pots for further examinations on the location until the next year. For our special question whether results on young spruces lead to conclusions about the vitality of the surrounding stock, root tips of nearby of old spruces are taken out for comparison. The so gained root tips are immediately put in well lockable vials (of about 10 ml contents) which contain aq. dest. and 1 drop of 1-Bromonaphthalene (Merck, Darmstadt). The vials containing the root tips and the solution are well shaken and then stored at room temperature. After 24 hours the samples are rinsed in tap water and fixed in ethanol : glacial acetic acid = 3 : 1 (v : v). Thus the specimens can be stored at a temperature of −18° C until their working up.

Staining of Nuclei

The fixed material must be well washed in aq. dest. and stained by Feulgen's reaction. For that matter the roots are hydrolized for three minutes at 63° C in 3 N HCl. The hydrolytic process is stopped by aq. dest. and the samples are put in Schiff's Reagents (after DRUŠKOVIČ 1987, 1988a, 1988b) and stained for about 30 minutes at room temperature.

Schiff's Reagents should always be made fresh and as follows: 2 g of Fuchsin (Bayer, Leverkusen) are added to 400 ml of quartz destilled water and the staining solution is boiled up. After cooling down to 50° C the solution is filtered and 60 ml 1 N HCl such as 7 g Potassiumbisulphate ($K_2S_2O_5$, Merck, Darmstadt) are added. This solution is stored in a dark place at room temperature for 24 hours and then mixed with 0.5 g activated charcoal (Merck, Darmstadt) and filtered. The pH is calibrated to 3.6 by means of 4% NaOH.

After staining the root tips are watered for at least ten minutes in aq. dest. From the thus treated root tips the meristem is now prepared under the stereomicroscope. The root is parted lengthwise and the stained meristem is parted, well squashed and observed in carmine acetic acid for the sake of mitosis stages under the light microscope. For observation and registration the slides are recorded by means of a video camera (Saba CVC 76 SL) and can be judged on a screen (Blaupunkt PM 40–48) simultaneously by several persons. Moreover it is possible thereby to record the objects by means of a videorecorder (Sony SLV 802) for examining them later.

Evaluation and Statistics

200 metaphases per tree of complete, not burst open cells are examined with regard to chromosome-aberrations (see results). In order to examine and classify abnormalities on chromosomes aberration index numbers are figured out. These aberration index numbers can be figured out by means of a control location, i. e. practically the place with the smallest number of abnormalities. The reason for taking the natural location with the smallest number of abnormalities lies in the fact that no completely unburdened location can be found in nature, resp. that a small share of chromosome abnormalities might even be found in completely unpolluted meristemes.

Example for figuring out the aberration index

A location shows an abnormality rate of 5%; i. e. 30 aberrations occur at 600 examined metaphases of one location (3 trees per location; 200 metaphases per tree) – whereby several abnormalities can be watched in one cell. If two different abnormalities occur. e. g. a break and a fragment, they are counted as two, whereas two homogeneous abnormalities in one cell are counted as one. The location with the lowest abnormalities (in our case 5%) gets the aberration index number 1. Another location shows a rate of 10% abnormalities which leads to an index of 2 (10 : 5 = 2).

By means of these aberration index numbers the locations can be graded in empiristic classes (cp. tab. 1, according to DRUŠKOVIČ 1988a) from 1 to 4 whereby 1 stands for the best and 4 for the worst class. Each class can furthermore be subdivided in a "plus" and a "minus" class. If the aberration index is grouped by the side to the next lower class, "plus" is valid, in case that the index comes close to the next higher class, "minus" is valid. All medium values of one class get no sign. The

above mentioned place with 10% abnormalities and aberration index 2 would have to be classified (according to tab. 1) in class 3 from four possible ones.

The statistic examination of the resulting factors was performed by means of the statistics programme NCSS from Unisoft Corp. for PC's. For evaluating the level of significance Fisher's LSD test was used (cp. WEBER 1967).

Table 1.

Class grading based on aberration index numbers (after DRUŠKOVIC 1988a) by considering better and worse aberration index numbers within one class. Explanation in the text.

aberrationindex	class
1.00 - 1.14	1
1.15 - 1.20	- 1
1.21 - 1.33	+2
1.34 - 1.47	2
1.48 - 1.60	-2
1.61 - 1.85	+3
1.86 - 2.10	3
2.11 - 2.35	-3
2.36 - 3.05	+4
> 3.06	4
< 3.06	

Results

When the roots were harvested in autumn and in spring (May, June, resp. September, October) it became evident that at most places the spruces had rooted well and showed many well-developed root tips. The roots grow along the inner side of the pot and can therefore easily be harvested by means of a pincette. From time to time we had to recognize a drop-out of spruces resp. no good rooting which can have different causes such as dry summers with dry ground. The reasons for an occasional strong mycosis of the root bulb have not yet been found. In these cases the formation of the roots is strongly reduced and such material should not be used as it shows only few metaphases. The surrounding screenwire protects the trees against trampling upon of animals and human beings thus preventing an additional drop out of sample trees.

For the question if results on young trees can also be transferred to old ones, root tips of old trees of the same location were also collected. A problem of harvesting roots of old trees is their widely ramified root-system which sometimes complicates the finding of root tips resp. the coordinating of roots to certain trees. Those roots of old trees which border on at the clay pot of the young spruce can be harvested easier but a definite coordination to a certain tree is not possible in most cases, as the roots cannot be traced back to the tree because of their length.

After the preparation (see material and methods – staining of nuclei) the meristeme cells are at hand separately, sometimes also one upon the other of mechanically destroyed, often with partly lacking cell wall (fig. 1). For observation, however, you can only take single cells which are completely surrounded by a cell wall. Mechanical destructions caused by certain shear forces at squashing, do also not exclude preparation-conditioned artefacts such as fractures on chromosomes. In case that the cell wall is completely intact, however, a destruction of chromosomes can be largely excluded. Interphase nuclei and all mitosis stages can be seen, whereby the highest number of metaphases of the mitosis stages is caused by the effects of 1-Bromonaphthalene. The chromosomes show a distinct pink colour and can be watched singly in the metaphase. The karyotype of *Picea abies* comprises $2n = 24$ chromosomes (fig. 2, fig. 3), whereby the description concerning the morphology of chromosomes is variable. Our investigations have revealed so far that further karyotypes can be expected. The observation of the metaphase-chromosomes is carried out in the light microscope at an 1000-old enlargement with immersion oil in the bright field, without filter, in order to watch and examine the single chromosomes properly. Other a bit smaller enlargements, like e. g. 800- resp. 650-fold, show the single chromosomes significantly, if they are situated within the metaphase plate, but these enlargements are not suitable for examining aberrations, because compounds, breaks and other abnormalities are hardly recognizable and furthermore metaphase-chromosomes which are lying in close contact to each other are endangered to get mixed up with stickiness. Filters resp. other methods by microscope (phase contrast, NOMARSKY interference contrast etc.) were tested, but they did not show any better contrast or any more significant presentation of the chromosomes. Microscope-photography resp. video-recording are used for recording the results. Microscope-photography serves the purpose of documentation, the video-recording is used for demonstration and it is helpful at open points (simultaneous discussion of several persons) and for storing datas, which later on also can be worked out further more via computer image analysis.

During the examination the following chromosome-aberrations can be observed and evaluated (classification modified according to DRUŠKOVIČ 1988a):

Specific aberrations: they concern abnormalities on single chromosomes.

Gaps (fig. 4)

Breaks (fig. 5)

Fragments (fig. 6)

Rings (fig. 7)

Unspecific aberrations: concerning the complete chromosome set.

Connections (fig. 8)

Stickiness (fig. 9)

Amorphous chromatin masses (fig. 10)

Further unspecific aberrations: they concern the complete chromosome set, but can only rarely be watched.

Fragmentation

Dissolution of chromatin

Investigations on chromosomes are also possible at different divisable tissues, such as leaf primordium or cambium. The first observations on chromosomes of leaf primordium were positive (unpublished). The chromosomes were well stained and the same chromosome-aberrations as with root meristemes were recognizable. The problem at the investigation on leaf primordium-chromosomes lies in the state of development. The cell divisions of this tissue are temporarily limited to such a degree, that it is extremely difficult to find the exact timing for collecting. Even between trees of one location there are great individual differences in sprouting and therefore naturally also in the mitosis phases. Furthermore, environmental factors as well as climatic shifts in different altitudes make an examination of leaf primordium chromosomes absolutely impossible. During examinations of the cambium old spruces from the stock of trees would have to be spot-drilled for getting investigation material. For all these reasons the meristemes from the roots are especially suitable, as they have a long growing period and they are easy to get from potted young spruces.

The proportion of specific to unspecific abnormalities varies strongly depending on the location (e. g. strongly sulphur-loaded locations show significantly more unspecific abnormalities than specific ones, non sulphur-loaded locations in inversion areas show quite a lot of specific aberrations – cp. also DRUŠKOVIČ (1988a, b). Referring to the question, whether the situation of young trees reflects the situation of old trees, we can say, as far as the number of chromosome-aberrations at young and old spruces from one location is concerned, that they are almost equal (cp. tab. 2). Regarding the kind of abnormalities at the locations which we examined in proportion of single chromosome aberrations versus abnormalities of the total chromosome set differences between young spruces and old spruces were distinguishable. On one hand young spruces often show a significant rise of the number of abnormalities of single chromosomes, and on the other hand with old spruces one can see a more frequent appearance of so-called unspecific

aberrations. As the abnormalities are valued equally and young and old trees show the same number of abnormalities, young and old spruces result in almost the same aberration index and therefore can be ranged in the same class (cp. tab. 1). First of all only the aberration index is decisive of the allotting to vitality classes. Further investigations will have to clarify the function of the single abnormalities.

Table 2.
Comparsion between the roots of young and old spruces of two locations. Explanation in the text.

locations and kind of trees	number of metaphases	number of aberrations (%)		
		specific	non specific	sum
1 young	400	8.5	2.5	11.0
1 old	200	3.5	7.0	10.5
2 young	100	12.0	5.3	17.3
2 old	400	6.3	8.5	14.8

Discussion

Picea abies is the most widely spread kind of tree in our area. It is very important for forestry and is therefore used as indicator tree. Because of its relatively large chromosomes and its delicate genetic material (REICHELT & KOLLERT 1985) the spruce is highly qualified for stating environmental influence.

The karyotype of *Picea abies* normally comprises 2n = 24 chromosomes (cp. MIYAKE 1903, SAX & SAX 1933, LÖVE & LÖVE 1961, SASAKI 1976, HIZUME 1988, PRICE 1989). Concerning the morphology of chromosomes, that means the position of the centromer, and the occurrence of secondary constrictions there are different specifications (DRUŠKOVIČ 1988a, TERASMAA 1971, 1972, 1975). Secondary constrictions generally are found in chromosomes II and III, more seldom in others (TERASMAA 1971, 1972, 1975).

DUMITRESCU 1972 examines the karyotype of different European spruce origins, especially from Rumania, and he states that there are significant differences concerning the relative length and the centromeric indices of chromosome arms. He can, however, not find a link to the geographic location of the provenances. Also in our examinations the existence of further karyotypes seems to appear in outlines. Changes in the length of chromosomes arise mainly from gamma – radiation (BEVILACQUA & VIDAKOVIĆ 1963) and from treatment with high temperatures (ERIKSSON & al. 1970).

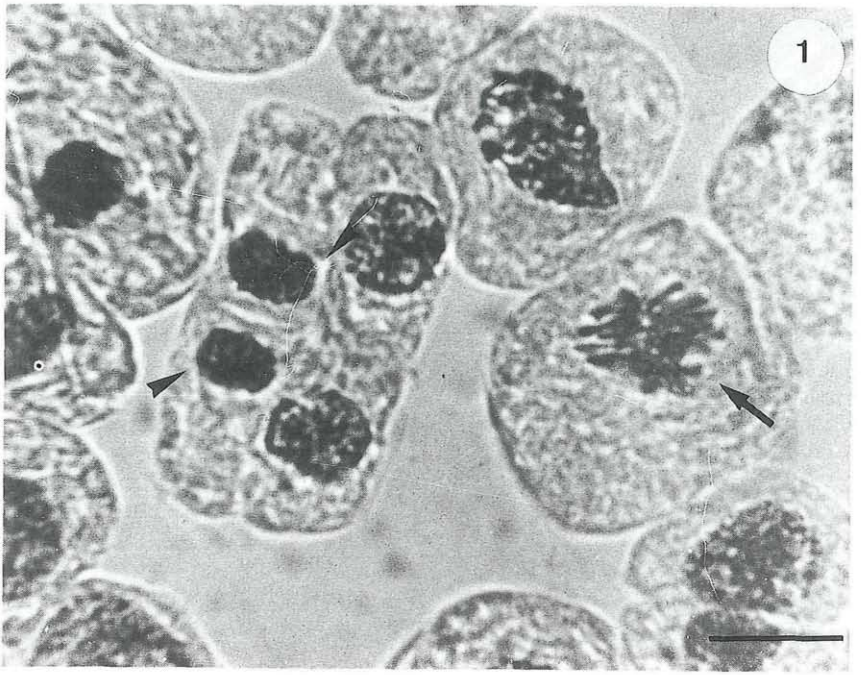
The chromosome aberrations at *Picea abies* resp. the abnormalities of chromosomes by which the chromosomes can be discerned from the normal

karyotype are examined on metaphase plates as abnormalities are most easily and effectively watched in this mitosis stage. The number of metaphases of a divisable tissue are highly increased by addition of a metaphase blocker (1 – Bromonaphthalene). These examined chromosome aberrations fundamentally differ from the ones of medical nomenclature, which among other things show no unspecific abnormalities of chromosomes (ISCN 1978). These abnormalities examined by us (divided into abnormalties of the whole chromosome set – unspecific aberration or abnormality of single chromosomes – specific aberration) can be watched frequently at plants and they are classified and described by several authors (e. g. SPARROW & WOODWELL 1963, KIHLMAN 1971, 1975, BAUCHINGER & al. 1972, RIEGER & al. 1973, GEBHART 1977, GRANT 1978, NILAN 1978, MOORE 1980, DRUŠKOVIČ 1988a, 1988b etc.).

During the investigations conducted up to now there has only been paid attention to the existence of any influence, i. e. whether there are any aberrations at chromosomes visible. The kind of chromosome abnormality (specific or unspecific – see results) cannot yet be ascribed to a certain influence. DRUŠKOVIČ 1988a not only differentiates between specific and unspecific abnormalities, but because of the relationship between specific and unspecific abnormalities she also offers a possibility of differentiation between acute and chronic influences. In this connexion she observes an increase of unspecific abnormalities by longterm influence. The degree of drawing a conclusion to a cause by the kind of chromosome abnormality is still uncertain. But there are numerous hints for such a possibility.

Our observations have shown that the method of "Cytogenetic Bioindication" is well applicable. The abnormalities are usually mainly visible at those locations, where raised sulphur concentration resp. also abnormalities in the tree crown are observable; at locations with low sulphur concentration resp. good tree crowns the results of the "Cytogenetic Bioindication" also show low aberration indices. An increased aberration index occurs more frequently however at locations with low sulphur concentration and tree crowns in good condition, while low aberration indices are evident at locations with raised sulphur concentration resp. tree crowns in bad condition. These forthleading factors, which are the subject of further investigations, are well considered by that method. The results gained so far by our group and by DRUŠKOVIČ (unpublished), also in combination with other investigation methods (e. g. sulphur analysis in needles, judging the shapes of tree crown condition) refer to the fact, that locations up to class plus 2 (cp. tab. 1) can be regarded as undamaged.

Concerning young and old trees there are almost no differences in the number of abnormalities, which is significant for our special question, but on the other hand there are differences concerning the kind of chromosome aberrations (cp. tab. 2). While young spruces of the location examined by us mainly show specific abnormalities, one can see with old spruces mainly



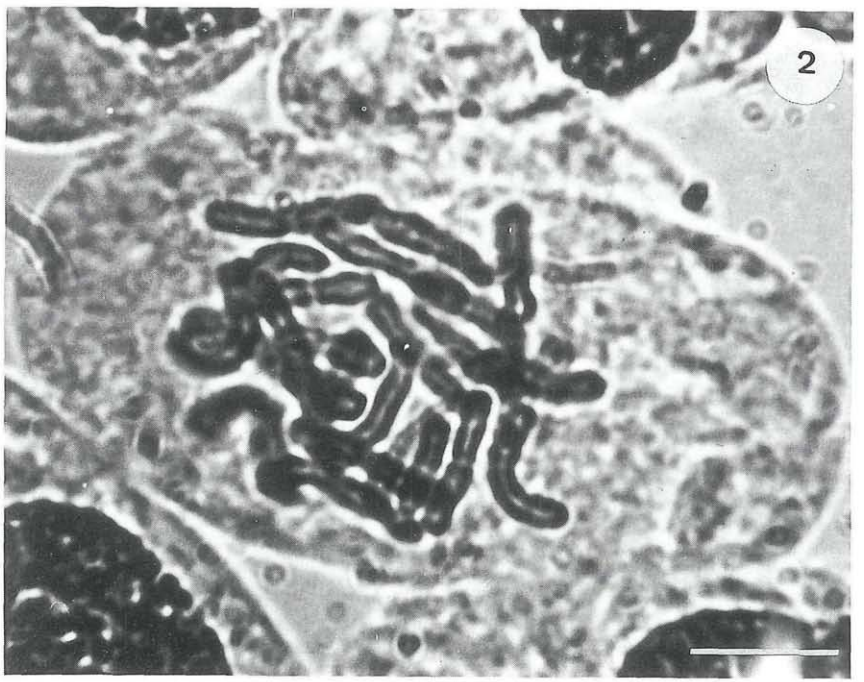
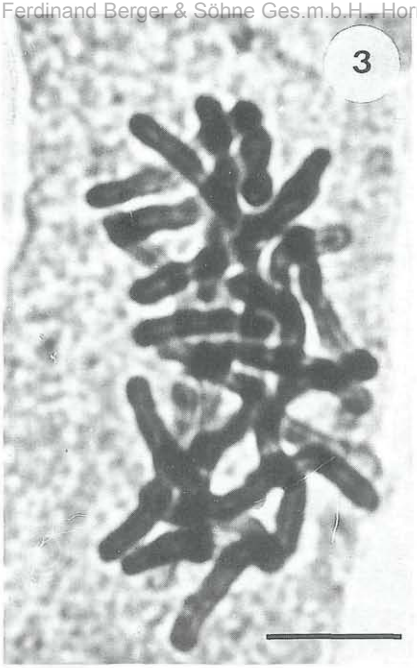
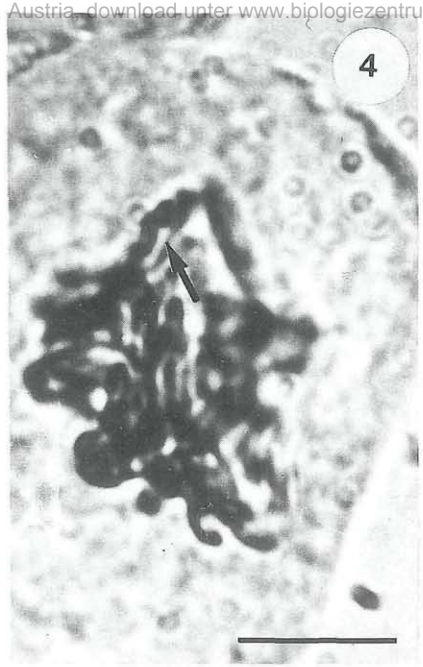


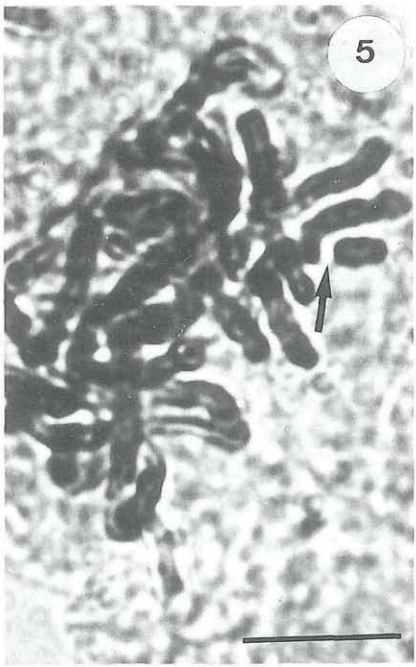
Fig. 1–10. Segments from the root meristem of *Picea abies* (L.) Karsten

Fig. 1. Survey of the root meristem; bar = 10 μm

Fig. 2. Metaphase plate; bar = 5 μm







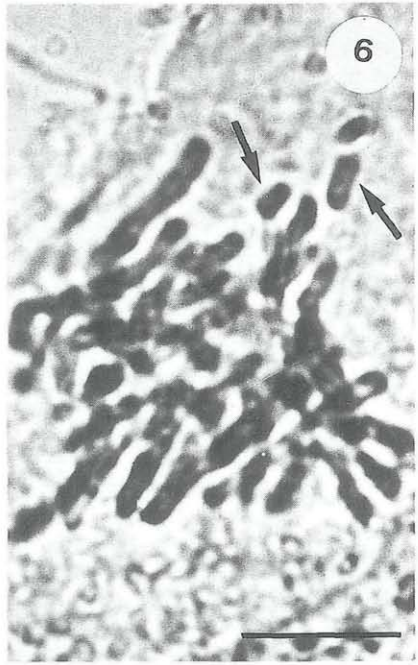
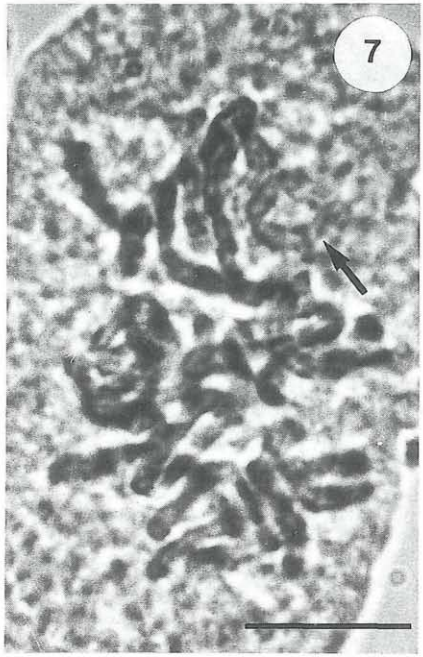


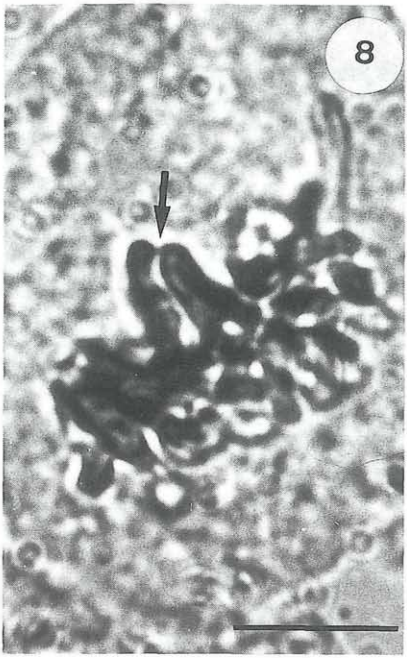
Fig. 3. Metaphase plate; bar = 5 μm

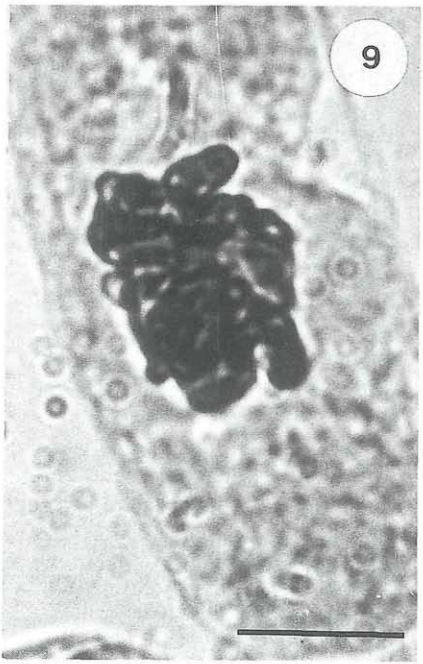
Fig. 4. Metaphase with one gap (arrow); bar = 5 μm

Fig. 5. Metaphase with one break (arrow); Bar = 5 μm

Fig. 6. Metaphase with two fragments (arrows); bar = 5 μm







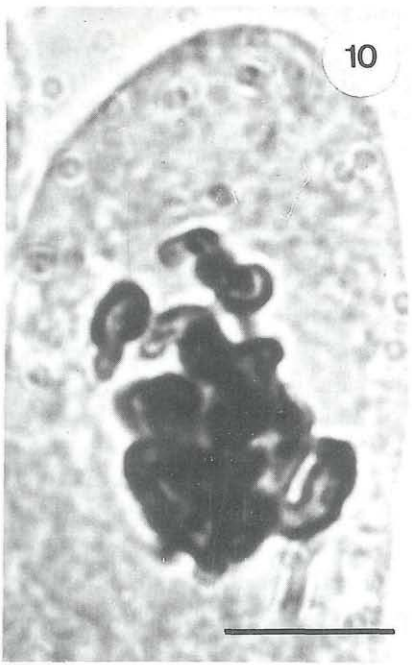


Fig. 7. Metaphase with one ring (arrow); bar = 5 μm

Fig. 8. Metaphase with one connection (arrow); bar = 5 μm

Fig. 9. Metaphase in stickiness; bar = 5 μm

Fig. 10. Metaphase as amorphous chromatin mass; bar = 5 μm

unspecific abnormalities, especially stickiness. Because of these results young and old spruces can be classified equally (cp. tab. 1).

So the results show no increase of the damage rate by potting spruces of the location and young trees near the ground allow correct conclusions to the old stock (see below). In contrast to the methods used so far it is therefore possible to use young spruces. The observations on young trees have turned out to be considerably easier and less complicated and they lead to a direct conclusion concerning the vitality of a location.

Summing up the results so far, the „Cytogenetic Bioindication" has proved to be a practicable method bringing forth completely new aspects concerning the determination of vitality. For its practical applicability, however, questions concerning the influence of natural stress of the ability of coordinating harmful influences on chromosome aberrations have to be clarified.

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