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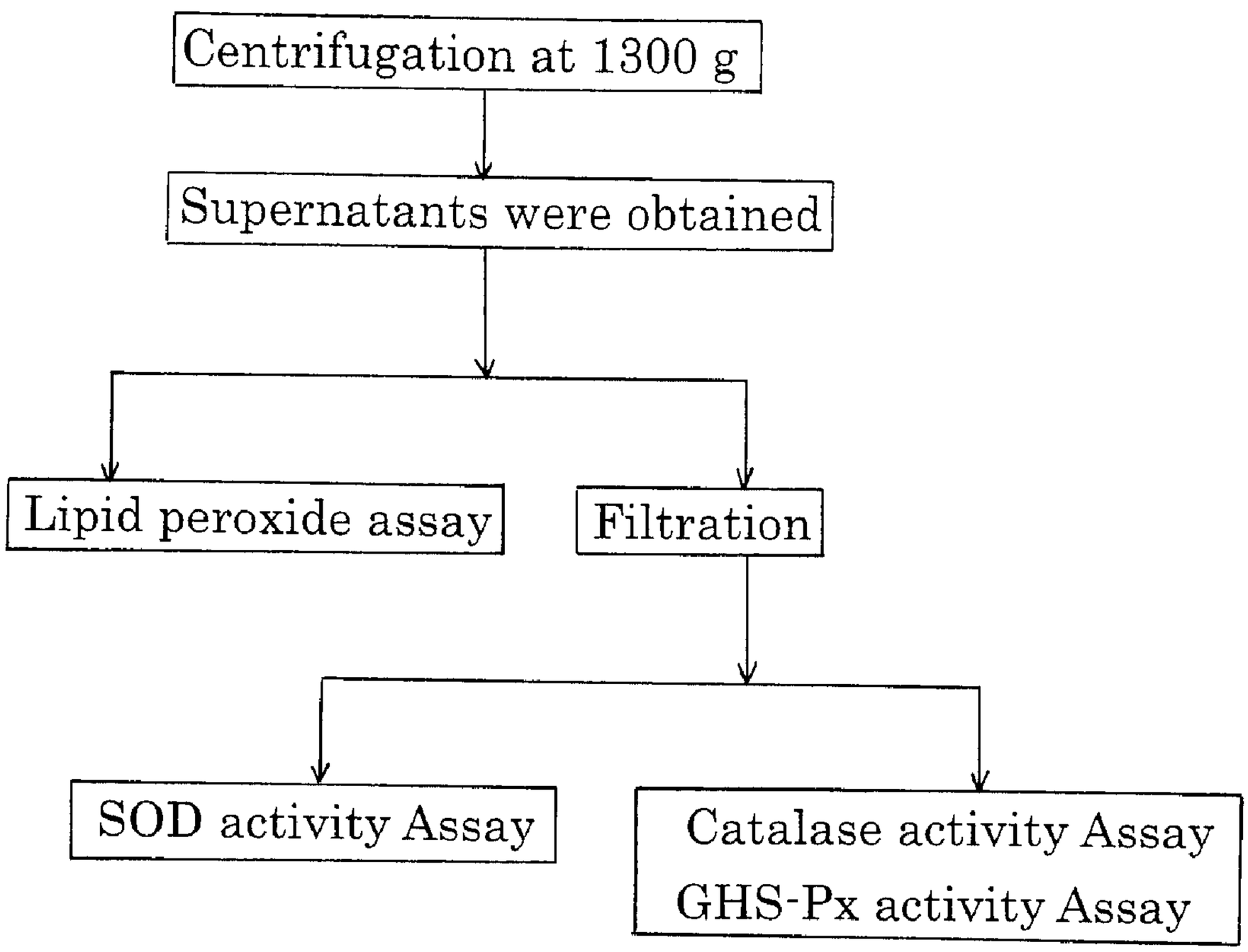


(57) Abrégé/Abstract:
The present invention provides a method of planting and growing trees which are resistant to ultraviolet rays. The methods according to the invention comprise the selection of plant species having triploid or tetraploid chromosomes.

ABSTRACT

The present invention provides a method of planting and growing trees which are resistant to ultraviolet rays. The methods according to the invention comprise the selection of plant species having triploid or tetraploid chromosomes.

# METHOD OF PLANTING TREES AND METHOD OF GROWING PLANTS

## BACKGROUND OF THE INVENTION

The present invention relates to a plant growing technique such as planting trees, and more specifically, to a technique of selectively growing young trees or the like which are resistant to ultraviolet rays so that vegetation such as a forest which is resistant to ultraviolet rays can be reliably obtained.

The problem of ultraviolet radiation has been the topic of recent discussion. Ultraviolet radiation is a serious concern especially in view of the destruction of the ozonesphere of the stratosphere surrounding the earth and the confirmation that ultraviolet radiation exposure is associated with negative biological impacts.

For example, with respect to human health, there have been reports of increased damage due to exposure to ultraviolet rays, and it has been confirmed that the increase in skin cancer cases is due to increased exposure to ultraviolet radiation. Damage resulting from exposure to ultraviolet radiation was thought to cause the occurrence of active oxygen. Niwa, one of the present inventors, has studied the pharmacological, biochemical properties of active oxygen and its damaging effects for many years.

## SUMMARY OF THE INVENTION

The current studies concerning the hazards of ultraviolet radiation has focused primarily on the deleterious effects of ultraviolet radiation exposure to the human body. In contrast, the present inventors have determined that ultraviolet radiation is very damaging to forests.

Previously, acid rain has been identified as the primary cause for forest

damage. For example, the widespread forest damage in the Schwarzwald district in Germany, has been reported. The report dealt with the damage to a large area of this district wherein many of the standing trees were whithered by acid rain. Acid rain is produced when acidic oxides such sulfur-containing oxides, which originate from industrial burning, are dissolved in rain drops.

The forest damage was investigated repeatedly by the present inventors who visited the damaged forest and obtained many results.

The present inventors observed the damage to the forest by repeatedly visiting several places of the damaged forest in several districts. After a detailed investigation of their observations, the present inventors investigated the possibility that factors other than acid rain were responsible for the withering of the trees in the forest. As a result of the present inventors' further research, exposure to ultraviolet radiation was found to be a main causative factor in the observed forest damage.

There have been no previous investigations on the role of ultraviolet radiation with respect to forest damage. In view of the present inventors' discovery of the damaging effects of ultraviolet radiation to forests, it is necessary to take rapid, practical measures to inhibit such forest damage. Because depletion of the ozonosphere and exposure to ultraviolet radiation is increasing on a global scale, urgent action should be taken to prevent ultraviolet radiation induced forest damage. If no action is taken, in the future, ultraviolet radiation damage may result in the disappearance of forests on a global scale.

The object of the present invention is to prevent forest damage caused by ultraviolet rays.

The artificially planted forest of the present invention is characterized in that it is constituted by planting seedling which are multiploid (TASUSBI in Japanese). The chromosome number of the seedlings is triploid or tetraploid. The seedlings are those of a conifer. The conifer seedlings are selected from a group consisting of Japanese cedar and Japanese cypress. The chromosome number of the seedling is obtained by increasing the number of chromosomes by breeding and mutation.

The method of planting trees of the present invention is characterized in that it comprises the steps of: selecting seedlings having multiploid chromosome; and planting the selected seedlings. The chromosome number of the seedlings is triploid or tetraploid. The seedlings are those of a conifer. The conifer seedlings are selected from a group consisting of Japanese cedar and Japanese cypress. The chromosome number of the seedlings is obtained by means of increasing the number of chromosomes such as breeding and mutation. The method of growing plants of the present invention is characterized in that it comprises the step of artificially forming vegetation which is significantly resistant to ultraviolet rays, as compared with vegetation constituted of diploid plants, by selectively growing plants whose chromosome number exceeds the basic number intrinsic of the species.

In this specification, "multiploid" (TASUSEI in Japanese) means a chromosome set having three or more times the usual number of chromosomes. Furthermore it should be noted that haploid, or diploid which is normally observed in plants, is not included into the scope defined by the aforementioned "multiploid".

## BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a flow chart showing the solution centrifugation procedure.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The seedling used in the artificially planted forest, method of planting trees and method of growing plants according to the invention is ultra-violet-resistant (including "UV-resistant" abbreviation afterwards). Afforestation according to the present invention is performed by choosing UV-resistant seedling to yield a artificially planted forest which is resistant to ultra-violet rays.

Upon selection of seedling possessing UV-resistant property, a standard of multiploid (TASUSEI) chromosomes acquired by the seedling is adopted for selection. The multiploid standard refers to seedling having more than triploid chromosomes.

The present inventors are the first to suggest the selection of UV resistant plants on the basis of chromosome number. Trees having multiploid chromosomes are found to be more resistant to the damaging effects of ultraviolet rays as compared to diploid trees. In the artificially planted forest formed by afforesting with UV-resistant seedling, the occurrences of damage from ultraviolet rays normally observed in the trees having diploid chromosomes can be inhibited effectively. Abnormal phenomena such as tree withering, evergreens losing their leaves, and deciduous trees retaining their leaves out of season are thought to be caused by ultraviolet rays, and thus can be inhibited.

The following illustrative examples describe the invention in further detail.

## EXAMPLES

In the Examples, the leaves of plants described in items (1)-(3) below, were collected and their ultraviolet resistance (UV-resistance) properties

investigated.

(1) Rice

For a comparison of samples, the rice leaf was used. Rice, a monocotyledonous plant, is well known for its resistance against ultraviolet rays. The rice leaf samples used in the experiment were grown at Tosashimizu city, Kochi Prefecture, Shikoku, Japan. The rice leaves were collected from five places at Tosashimizu city and enzyme activity, etc. was measured by the methods described later.

(2) Osmunda

Leaves of osmunda which were spontaneously growing in Tosashimizu, Japan, were collected as the object of experiment. It is well known that osmunda, a fern, is evolutionarily older than other trees. There are many reports which disclose the ability of osmunda to adapt to the environmental conditions by increasing its chromosome number.

(3) Gymnosperms such as Black Pines etc.

The leaves of gymnosperms were included in the experiments as these plants are sensitive to ultraviolet rays. As described in Tables 1-4 of this Example, leaves from KUROMATSU trees (English name: Japanese black pine tree), SUGI trees (English name: Japanese cedar) and HINOKI trees (English name: Japanese cypress) which were spontaneously growing in mountains of Japan, were included in the experiments. The black pine, cedar and cypress leaf samples were collected from various places as shown in Tables 1-4 and numerated as shown in Tables 1-4.

Assays for SOD activity, GSH-Px activity and lipid peroxide levels were performed on black pines (hereinafter abbreviated as "control black pine) known to be sensitive to common UV rays and on black pines resistant to UV

rays (hereinafter abbreviated as "UV-resistant black pine). The results are described in Table 1,

Furthermore, as demonstrated in Tables 5 and 6, measurements for triploid species and tetraploid species resulting from crossing and mutation were also performed.

[Table 1] Comparison of lipid peroxide levels, SOD and glutathione peroxide (GSH-Px) activities between UV resistant Japanese black pines and UV sensitive Japanese black pines.

| | Sample name | SOD activity (unit/gr) | GSH-Px activity (unit/gr) | Lipid peroxide Levels (n mol/gr) |
|---|---|---|---|---|
| UV resistant Japanese black Pines | Nakamikata -73 | 391±46 | 0.0215±0.0032 | 18.0±2.1 |
| | Tosashimizu -63 | 225±29 | 0.0171±0.0021 | 16.7±2.0 |
| | Ooita -8 | 201±28 | 0.0269±0.0029 | 18.2±2.3 |
| | Ei -425 | 358±46 | 0.0190±0.0022 | 20.7±2.8 |
| | Tsuyasaki-50 | 461±73 | 0.0236±0.0035 | 22.2±3.3 |
| | Misaki -90 | 346±41 | 0.0230±0.0036 | 23.4±3.0 |
| | Namikata- 73 | 456±59 | 0.0331±0.0052 | 22.0±3.7 |
| | Tanabe -54 | 367±44 | 0.0214±0.0027 | 20.5±2.8 |
| | Yasu -37 | 195±21 | 0.0236±0.0033 | 18.4±2.9 |
| | Yoshida -2 | 817±106 | 0.0246±0.0039 | 23.5±2.8 |
| | Sendai -290 | 245±31 | 0.0233±0.0039 | 19.9±2.7 |
| | Mitoyo -103 | 896±107 | 0.0276±0.0030 | 24.1±3.8 |
| | Average | 423±56* | 0.0237±0.0035 | 20.6±3.6 |
| Control UV sensitive Japanese black pines | No.1 | 252±30 | 0.0177±0.0023 | 20.5±3.2 |
| | No.2 | 99±11 | 0.0142±0.0018 | 22.9±4.1 |
| | No.3 | 141±18 | 0.0198±0.0031 | 21.1±2.9 |
| | No.4 | 356±46 | 0.0206±0.0037 | 21.9±3.5 |
| | No.5 | 352±42 | 0.0198±0.0017 | 18.7±2.4 |
| | Average | 240±37* | 0.00184±0.0031 | 21.0±3.8 |

*P<0.01 between UV resistant and sensitive black pine trees.

[Table 2] Comparison of lipid peroxide levels, SOD and GSH-Px activities in Japanese cypress triploid

| | | Sample name | Poly ploidy | SOD activity (unit/gr) | GSH-Px activity (unit/gr) | Lipid peroxide Levels (n mol/gr) |
|---|---|---|---|---|---|---|
| Japanese cypress | Control diploids | B-4 | 2X | 472±56 | 0.0180±0.0023 | 19.9±2.3 |
| | | B-11 | 2X | 614±73 | 0.0155±0.0020 | 21.3±2.9 |
| | | B-13 | 2X | 552±71 | 0.0019±0.0003 | 27.5±3.0 |
| | | D-4 | 2X | 874±104 | 0.0107±0.0016 | 26.4±3.9 |
| | | D-9 | 2X | 549±65 | 0.0028±0.0003 | 25.7±3.5 |
| | | D-12 | 2X | 683±88 | - | 28.8±3.7 |
| | | D-14 | 2X | 466±60 | 0.0077±0.0012 | 22.9±3.6 |
| | | Average | | 601±46 † | 0.0094±0.0002 | 24.6±4.1 |
| | Artificial Triploids | A-8 | 3X | 479±71 | 0.0123±0.0017 | 24.7±3.4 |
| | | A-13 | 3X | 543±70 | 0.0119±0.0015 | 27.8±3.6 |
| | | A-24 | 3X | 685±89 | 0.0014±0.0002 | 28.1±4.4 |
| | | A-54 | 3X | 505±70 | 0.0058±0.0008 | 22.7±3.1 |
| | | A-58 | 3X | 558±55 | 0.0079±0.0012 | 21.1±2.3 |
| | | A-72 | 3X | 674±74 | - | 19.1±2.4 |
| | | A-77 | 3X | 482±77 | 0.0065±0.0011 | 20.8±3.3 |
| | | C-6 | 3X | 559±61 | 0.0100±0.0012 | 21.5±2.5 |
| | | C-22 | 3X | 548±65 | 0.0126±0.0017 | 19.5±2.3 |
| | | C-37 | 3X | 580±69 | 0.0055±0.0008 | 22.2±2.8 |
| | | C-50 | 3X | 589±82 | - | 21.1±3.1 |
| | | C-55 | 3X | 577±75 | - | 20.9±2.7 |
| | | C-60 | 3X | 624±74 | - | 19.4±2.3 |
| | | C-140 | 3X | 646±83 | 0.0026±0.0004 | 19.9±2.7 |
| | | C-225 | 3X | 1005±120 | | 21.1±2.1 |
| | | Average | | 606±51 † | 0.0076±0.0018 | 22.0±3.9 |

[Table 3] Comparison of lipid peroxide levels, SOD and GSH-Px activities in plus trees of Japanese cypress

| | | Sample Name | Poly-ploidy | SOD activity (unit/gr) | GSH-Px (unit/gr) | Lipid Peroxide Levels (n mol/gr) |
|---|---|---|---|---|---|---|
| Japanese Cypress (Plus Trees) | Diploids | Aso-7 | 2X | 459±55 | 0.0069±0.0009 | 24.4±2.9 |
| | | Saeki-17 | 2X | 423±54 | 0.0160±0.0020 | 21.2±2.7 |
| | | Hiji-4 | 2X | 544±76 | 0.0019±0.0002 | 23.5±2.5 |
| | | Kusu-6 | 2X | 459±59 | 0.0171±0.0027 | 20.7±2.4 |
| | | Taketa-8 | 2X | 655±85 | 0.0068±0.0009 | 24.0±2.6 |
| | | Average | | 508 ± 78 † | 0.0097±0.0018 | 22.7±4.3 |
| | Triploids | Misoshi-4 | 3X | 420±46 | 0.0136±0.0020 | 23.1±2.3 |
| | | Fuji-2 | 3X | 582±75 | 0.0106±0.0012 | 14.1±1.5 |
| | | Average | | 501 ± 76 † | 0.0121±0.0026 | 18.6±4.1 |
| | Tetraploid | Sanko-Hinoki | 4X | 1902±190 | 0.0103±0.0012 | 21.5±3.4 |
| | | Kiso-4X | 4X | 905±99 | 0.0058±0.0007 | 16.2±2.5 |
| | | Average | | 1403 ± 212 † | 0.0081±0.0013 | 18.8±2.8 |

† P<0.001(between tetraploids of plus trees of Japanese and diploids or triploids ofJapanese cypress, between tetraploids of plus trees of Japanese and diploids or triploids of plus trees of Japanese cypress)

[Table 4] Comparison of lipid peroxide levels, SOD and GSH-Px activities in plus trees of Japanese cedar, rice leaves and osmund

| | | Sample Name | Poly-ploidy | SOD activity (unit/gr) | GSH-Px activity (unit/gr) | Lipid Peroxide Levels (n mol/gr) |
|---|---|---|---|---|---|---|
| Japanese Cedar (Plus Trees) | Diploids | Naga-9 | 2X | 12±1.4 | 0.0134±0.0021 | 4.6±0.5 |
| | | Akita-1 | 2X | 37±4.4 | 0.0096±0.0015 | 5.0±0.6 |
| | | Saga-3 | 2X | 21±2.5 | 0.0114±0.0018 | 3.1±0.5 |
| | | Saeki-10 | 2X | 52±6.7 | 0.0104±0.0015 | 4.1±0.5 |
| | | Kunisaki-3 | 2X | 26±2.8 | 0.0104±0.0017 | 4.5±0.7 |
| | | Nishikawa-2 | 2X | 17±2.2 | 0.0093±0.0011 | 5.0±0.8 |
| | | Average | | 27 ± 3.8 § | 0.0108±0.0019 | 4.3±0.6 |
| | Triploids | Ooi-5 | 3X | 161±19 | 0.0141±0.0019 | 7.0±0.8 |
| | | Kuji-30 | 3X | 41±5.3 | 0.0092±0.0014 | 3.7 ± 0.4 |
| | | Onta-2 | 3X | 317±44 | 0.0150±0.0024 | 9.5 ± 1.1 |
| | | Kyurin-3X | 3X | 108±11 | 0.0182±0.0023 | 8.7 ± 1.1 |
| | | Fujitsu-28 | 3X | 236±28 | 0.0158±0.0022 | 8.2 ± 1.2 |
| | | Miyoshi-10 | 3X | 95±12 | 0.0109±0.0011 | 6.1 ± 0.8 |
| | | Kuga-1 | 3X | 59±8.2 | 0.0115±0.0013 | 8.0 ± 1.2 |
| | | Average | | 145±23 ‡ | 0.0135±0.0021 | 7.3 ± 1.2 |
| | Tetraploid | Kanagawa-sugi | 4X | 3589±430 | 0.0176±0.0017 | 8.2 ± 0.9 |
| | | Cr-38 | 4X | 2684±295 | 0.0128±0.0020 | 9.3 ± 1.2 |
| | | Average | | 3136±40 ‡ § | 0.0152±0.0025 | 8.7 ± 1.3 |
| rice leaves | | Tosashi-mizu | | 2941±352 | 1.3841±0.1522 | 71 ± 8.5 |
| Osmund | | Tosashi-mizu | | 1394±181 | 0.0889±0.0115 | 51 ± 8.1 |

‡ P<0.0001 between tetraploid and triploids of plus trees of Japanese cedar.
§ P<0.00001 between tetraploids and diploids of plus trees of Japanese cedar.

As shown in Table 1, ultraviolet-resistant (UV-resistant) Japanese black pines were compared with ultraviolet-sensitive Japanese black pines (control) with respect to SOD activity, glutathione peroxidase (GSH-Px) activity and lipid peroxide levels.

As shown in Table 2, diploids of Japanese cypress were compared with triploids with respect to lipid peroxide levels, SOD activity and GSH-Px activity.

As shown in Table 3, diploids, triploids and tetraploids of plus trees of Japanese cypress were compared with respect to individual SOD activity, GSH-Px activity and lipid peroxide levels.

As shown in Table 4, SOD activity, GSH-Px activity, lipid peroxide level were compared for diploids, triploids and tetra-ploids of plus trees of Japanese cedar, rice leaves and osmunda. The comparison of Table 4 with Tables 5 and 6 are described later.

The extracts from the leaves of diploid, triploid and tetraploid species of Japanese cedar and Japanese cypress were compared in Table 5 and 6 as described later. As shown in Table 5, in the column titled "sample added", the term "x1" refers to a sample size of 0.6gr/ml. Accordingly, the term "x5" refers to a sample size of 3.0gr/ml and "x10" refers to a sample size of 6.0 gr/ml. In Table 6, the data of rice leaves are included for comparison.

The individual enzyme activity and lipid peroxide level for the plants described in items (1)-(3) were assayed. Enzyme activity was determined by measuring: SOD activity, glutathione peroxidase (GSH-Px) activity and catalase activity. The results obtained are shown in Tables 1-4. The results are given in terms of mean ± standard deviation (SD). Catalase activity for those plants other than rice leaves was utterly not detectable and their results were not shown in Tables 1-4.

Quantitative analysis of lipid peroxide level was performed by,measuring lipid peroxide levels using the TBA reaction method of

converting docohexanoic acid into a TBA reactive substance.

The leaf homogenates from the individual plants described above were prepared as follows and used as samples. Leaves collected were from the plants described in Items (1)-(3). The collected leaves were powdered with a mill. The leaf homogenates from individual plants described above were used as samples. The procedure for preparation of the leaf homogenates is as follows. The powder was suspended in 95% ethanol solution for lipid peroxide formation test or in physiological saline for those tests other than lipid peroxide formation test. In both cases, the concentration of the suspensions was 60 mg/ml. The suspension was then sonicated at 15W for 15 seconds.

After sonication, the solution was centrifuged (at 1300 g). The supernatants after centrifugation were divided into two parts for use as samples for the procedures shown in FIG. 1,. One part of the supernatant was used for the preparation of samples for lipid peroxide level determination. The other part was filtrated through a 45 μm filter. One portion of the filtrate was used as sample for SOD activity assay and the remaining filtrate was used as sample for catalase, GSH-Px activities assays.

SOD's Quantitative Analysis

The mixture prepared as described above, was added to 0.2 ml of an assay mixture containing xanthine-xanthine oxidase producing $O_2$ and the resulting $O_2$ quantity was determined from the reduction quantity of ferri-cytochrome c (type III) as determined by the measurement of optical absorbance at 550 nm with a spectrophotometer (Beckman).

The quantity of SOD contained in sample is defined as the reduction quantity of reducing cytochrome c at 50% and 1 unit of SOD is expressed in terms of unit/mg protein. In this analysis, the sample itself not only inhibited the reaction on cytochrome c without the mediation of $O_2$, a small quantity of cytochrome c would be directly reduced. With these factors, the real SOD quantity was calculated by the formula:

unit={a-(b-c)}/(a/2)

where

a: absorbance obtained by the addition of xanthine oxidase alone.

b: absorbance obtained by leaf homogenates in the presence of xanthine oxidase.

c: absorbance obtained by the addition of leaf homogenate alone before the addition of xanthine oxidase.

Catalase Activity Assay

The catalase activity was determined by measuring the velocity of reduction of hydrogen peroxide ($H_2O_2$). The velocity of reduction was measured for 12-30 seconds in the presence of leaf homogenate samples, using a spectrophotometer at 240 nm. Activity was expressed with the formula shown below.

K=(2.3/18) X log(A1/A2)

A1: Absorbance measured for 12 seconds at 240 nm for the assay mixture of total volume at 3.1 ml consisting 10 mmol/L H202 dissolving in 3 ml of 50 m mol/L potassium phosphate buffer solution and 0.1 ml of leaf homogenate.

A2: Absorbance measured for 30 seconds at 240 nm for above assay mixture

In this assay, sodium perborate was used as the substrate as hydrogen peroxide is potentially instable. In this case, 0.002-0.05 ml of the leaf sample solution was suspended in 2.8 ml of 0.05 mol/L potassium phosphate buffer solution (pH 7.4) and the mixture was preincubated at 30°C for 5 minutes. Thereafter, a 0.2 N sodium perborate solution was added to a cuvette containing the preincubated mixture and the reaction was started. The data was recorded for 2-3 minutes at 220 nm.

GSH-Px Activity Assay

For GSH-Px measurement, the method of Lawrence and Burk, oxidation of NADPH by glutathione reductase, was used. The oxidation potency for NADPH was measured at 37°C with a spectrophotometer at 340 nm.

The assay mixture consisted of 50 mmol/L potassium phosphate buffer solution (pH 7.0), 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L NaN3, 0.2 mmol/L NADPH, 1 mmol/L glutathione, 2 unit of glutathione reductase, and 1.5 mmol/L cumene hydroperoxide or 10 mmol/L tert-butylperoxide solutions.

To the above mixture, leaf homogenate was added to make the resulting solution volume 1.0 ml. The enzyme activity measured by this method is shown as the quantity of NADPH oxidized per minute. The activity is expressed in terms of unit/mg protein according to Lowry's method.

Lipid Peroxide Assay

The polyunsaturated fatty acid 4,7,10, 13, 16, 19-docosahexanoic acid was diluted to 200-fold in 94% ethanol. To the test tube containing this substrate, various concentration of leaf homogenate was added and subjected to radiation with ultraviolet rays. In the experiment, homogenates of 0.6, 3.0 and 6.0 mg/ml concentration were tested. After UV irradiation, the solution was analyzed by fluorospectrometry to measure the quantity of TBA (thiobarbituric acid) using a Hitachi F-2000 fluorospectrophotometer with the excitation wave length set at 515 nm for and the emission wave length set at 553 nm.

The result is shown as mean ± standard error (S.E.). The quantity of TBA substance was adopted as the lipid peroxide quantity. Furthermore, significant difference analysis was performed using the Student t-test. The result of t-test demonstrates the significant difference between the two groups.

Identification of Ploids of Chromosome

Poloidy of the previously described trees was determined by cytological analysis using root ends cells or by flowcytometric analysis using leaf cells.

Discussion on Tables 1-6, was performed as follows. As shown in Tables 1-4, rice and osmunda leaves (ref. Table 4) showed an apparent high SOD

activity as compared to gymnosperms plants such as Japanese black pines, Japanese cedar, Japanese cypress. In particular the GHS-Px activity of rice leaf was as high as 1.384 unit/g and SOD activity as high as 2941 unit/g.

As can be seen from Table 4, the SOD activity for rice leaf is higher than those of the individual trees tested (except Japanese cedar, a tetraploid plant), and in some cases, showed the highest SOD activity with a 100-fold difference in activity as compared to the individual ordinary trees tested. As seen in Table 4, osmund leaf also showed SOD activity as high as 1394 unit/g.

Table 1 ($P<0.01$) shows the assay results for the gymnosperms plants. The SOD activity for Japanese black pines possessing ultraviolet-resistant property was found to be apparently higher than the ordinary Japanese black pines. For example, the SOD activity of the Japanese black pines possessing ultraviolet-resistant property is 413± 56 unit/g on average, and it is much higher than the measured average SOD activity of 240±37 unit/g of the ordinary Japanese black pines.

From the above results, it is ascertained that trees possessing the ultraviolet-resistant properties, also have high SOD activity. This may suggest that SOD activity plays a role in protecting the trees from the damage caused by ultraviolet rays.

When comparing the SOD activity of triploid species tree and tetraploid species tree, as shown in Tables 2-3, especially in the individual trees, the tetraploid species tree showed a higher SOD activity than triploid species tree. The plus tree of the Japanese cedar (sample name Kamikawa cedar, Cr-38c) as shown in Table 4, showed a very high SOD activity as compared to ordinary trees. In such Japanese cedar, the apparent increase of SOD activity was found in trees possessing tetraploid chromosome. In contrast to this, both diploid and triploid trees showed no apparent increase of SOD activity.

Secondly, as to the lipid peroxide level, it was demonstrated that those cedars having a small quantity of resin had very little lipid peroxide formation

ability as compared with other trees.

No difference in GSH-Px activity between ultraviolet-resistant Japanese black pines and ultraviolet-sensitive ordinary Japanese black pines (used as a control) in was not found (refer to Table 1, $P>0.05$). This may suggest that there is no relationship between GSH-Px enzymes and ultraviolet resistance.

[Table 5] Comparison of SOD activity and the effect of lipid peroxidation by the extract of leaves among 2X, 3X and 4X in Japanese trees

| Samples | Polyploidy | SOD Activity (unit/gr) | Lipid peroxidation | |
|---|---|---|---|---|
| | | | average | |
| | | | UV(-) control 45 | |
| | | | UV(+)control 260 | |
| | | | Sample added | % control |
| S-20 | 2X | 133±15 | x1*(0.6mgr/ml) | 124.6±14.9 |
| | | | x5*(3.0mgr/ml) | 73.3±9.5 |
| | | | x10*(6.0mgr/ml) | 33,8± 4.7 |
| S-22 | 2X | 149±17 | x 1 | 115.3±13.8 |
| | | | x 5 | 71.4± 9.2 |
| | | | x10 | 31.5± 4.4 |
| S-23 | 2X | 421±54 | x 1 | 221.5±24.3 |
| | | | x 5 | 137.9±17.9 |
| | | | x10 | 60.3± 7.2 |
| S-25 | 3X | 629±81 | x 1 | 174.6±19.2 |
| | | | x 5 | 112.5±14.6 |
| | | | x10 | 63.9±10.2 |
| S-26 | 3X | 2173±260 | x 1 | 82.7±10.7 |
| | | | x 5 | 62.1± 9.9 |
| | | | x10 | 43.6±5.2 |
| S-28 | 3X | 309±33 | x 1 | 150.6±24.0 |
| | | | x 5 | 89.2±13.3 |
| | | | x10 | 41.9± 6.7 |
| S-30 | 4X | 1382±193 | x 1 | 84.2±10.9 |
| | | | x 5 | 62.3± 8.7 |
| | | | x10 | 42.7± 4.6 |
| S-31 | 4X | 1447±231 | x 1 | 79.4± 10.3 |
| | | | x 5 | 57.3± 9.1 |
| | | | x10 | 37.1± 5.1 |
| S-32 | 4X | 1096±164 | x 1 | 72.9± 9.4 |
| | | | x 5 | 61.4± 7.9 |
| | | | x10 | 51.9± 8.3 |
| S-33 | 4X | 1237±160 | x 1 | 71.6±12.1 |
| | | | x 5 | 49.8± 6.4 |
| | | | x10 | 38.4± 6.1 |
| S-34 | 4X | 1645±213 | x 1 | 86.0±11.1 |
| | | | x 5 | 64.1± 9.6 |
| | | | x10 | 44.1± 7.0 |
| S-36 | 4X | 1105±154 | x 1 | 140.1±15.4 |
| | | | x 5 | 94.8±15.1 |
| | | | x10 | 53.7± 6.9 |
| S-38 | 4X | 1046±135 | x 1 | 136.6±19.1 |
| | | | x 5 | 111.6±15.6 |
| | | | x10 | 89.0±10.6 |

*x1 denotes 0.6 gr/ml as unit and the column "sampl added" shows multiple of theunit

[Table 6] Comparison of SOD activity and the effect of lipid peroxidation by the extract of leaves among 2X, 3X and 4X in Japanese trees

| Samples | Polyploidy | SOD activity (unit/gr) | Lipid peroxidation | |
|---|---|---|---|---|
| | | | Average | |
| | | | UV(-) control 45 | |
| | | | UV(+)control 260 | |
| | | | Sample added | % control |
| H-1 | 2X | 160±19 | x1*(0.6mgr/ml) | 163.0±17.9 |
| | | | x5*(3.0mgr/ml) | 98.2±11.7 |
| | | | x10*(6.0mgr/ml) | 58.5± 7.6 |
| H-2 | 2X | 352±45 | x 1 | 151.8±21.2 |
| | | | x 5 | 103.4±11.3 |
| | | | x10 | 56.8± 6.2 |
| H-5 | 3X | 578±80 | x 1 | 170.0±22.1 |
| | | | x 5 | 114.3±17.1 |
| | | | x10 | 60.8± 7.2 |
| H-6 | 3X | 1066±138 | x 1 | 95.2±13.3 |
| | | | x 5 | 53.1± 6.3 |
| | | | x10 | 45.2± 5.8 |
| H-7 | 3X | 742±89 | x 1 | 139.1±15.3 |
| | | | x 5 | 87.6±10.5 |
| | | | x10 | 40.4± 4.4 |
| H-8 | 3X | 821±106 | x 1 | 123.6±14.8 |
| | | | x 5 | 78.4± 9.4 |
| | | | x10 | 38.1± 4.9 |
| H-9 | 3X | 1280±140 | x 1 | 92.5±12.9 |
| | | | x 5 | 62.3± 6.9 |
| | | | x10 | 39.0± 4.6 |
| H-10 | 4X | 1773±265 | x 1 | 52.6± 6.8 |
| | | | x 5 | 37.8± 6.0 |
| | | | x10 | 25.3± 3.5 |
| H-11 | 4X | 1232±172 | x 1 | 90.8±12.7 |
| | | | x 5 | 61.4± 7.9 |
| | | | x10 | 32.3± 4.8 |
| rice leaves | | 2941±352 | x 1 | 455.2±54.6 |
| | | | x 5 | 298.3±38.7 |
| | | | x10 | 165.0±21.4 |

*x1 denotes 0.6 gr/ml as unit and the column "sampl added" shows multiple of the unit

Lipid peroxide formation was enhanced at in samples with low concentrations of homogenate (e.g. 0.6 mg/ml) whereas samples with high concentrations of homogenate inhibited the formation of lipid peroxide. This is clearly shown in Tables 5 and 6.

As shown in Tables 5 and 6, when tetraploid tree leaf samples are compared with each other, it is clear that in those samples having high SOD activity strongly inhibit lipid peroxide formation even in presence of low sample concentrations. This may suggest that SOD activity plays a cytotoxic role by inhibiting formation of lipid peroxide. This means that the higher SOD activity, the more extensive the inhibition on the lipid peroxide formation.

For example, Japanese cedar sample nos. S-26, -30, -32, -34, -36-38 in as show in Table 5 and Japanese cypress samples nos. H-6, -9, -10, -11 as show in Table 6, have higher SOD activity than 1,000 unit/g. Among them, nine cases: sample nos. S-26, -30, -32, -34; H-6, -9, -10, showed a lower lipid peroxide formation (indicating in column of x1 of Table 5) with the addition of low sample concentration as compared to the controls.

In the case of rice leaf samples, the formation of lipid peroxide was inhibited regardless of whether a low concentration of homogenate (see "x1 column" of Table 5) or high concentration (see "x10 column" of Table 5) was used. Thus, the SOD activity demonstrated a high activity as those of GHS-Px activity and catalase activity. Among the leaf samples tested, the rice leaf showed the highest level for lipid peroxide formation. For samples other than rice leaf, their catalase activities were not detectable and thus data are not included in Tables.

On the other hand, the higher SOD activity, the stronger anti-oxidation effects are known as usual. The SOD plays an important role of removal of active oxygen.

In considering the factors involved in the formation of active oxygen, the existence of ultraviolet rays was not neglected. In recent years,

destruction of the ozonosphere has resulted in increased ultraviolet radiation reaching the earth. Based on this evidence, an apparent increase of oxygen radical had been identified. Like human beings, trees are damaged when exposed to ultraviolet rays and have a high concentration of oxygen radical.

In view of the experimental results obtained by the present inventors which show high SOD activity for those trees possessing ultraviolet resistance, it is believed these trees protect themselves from damage exerted from environment by using the anti-oxidization agents such as SOD containing in their leaves. Until now, there have been no reports concerning the self-protection against the environmental damage, by trees using anti-oxidization agent possessed in the trees. This is the new finding by the present inventors.

The anti-oxidization ability of ultraviolet-resistant pine trees (hereinafter abbreviated as "UV-resistant") was compared with that of ordinary pine trees. Usually, GHS-px activity is induced in response to oxygen radical. However, in the experiments performed by the present inventors, there was no apparent change of GHS-px activity which paralleled to SOD activity.

With respect to catalase, there was a report dealing with of the induction of catalase by oxygen radicals in a tree. But in the present experiment, the inventors found no occurrence of catalase activity except for rice leaves. The lack of catalase activity may be due to compounds such as terpene, etc in the homogenates which inhibit catalase activation.

It is well known that like the enzymes such as superoxidase and low molecular weight substances for example, vitamin C an anti-oxidizing agent, play a major role in trees. The inventors did not investigate the anti-oxidization function of low molecular weight compounds but did investigate the anti-oxidization functions of SOD. It is concluded that SOD, a strong anti-oxidizing enzyme occurring in tree leaves, protects the tree from against excessive oxygen radicals.

Further to the above discussions, when considering the relationship of SOD activity and chromosome number of plants, those trees possessing triploid and tetraploid, and especially the tetraploid trees, have a high SOD activity as discovered by the present inventors. There have been no discussion on the relationship between extent of SOD activity and ploid of tree done by others, and thus the results obtained by the present inventors as mentioned above are utterly beyond estimation.

When considering the relationship between SOD activity and multiploid of chromsome, it appears that an increase in the number of chromosomes encoding the SOD gene, results in an increase in the amount of SOD. This hypothesis is supported by evidence of increased SOD in human beings with additional chromosomes. For example, people with Down's syndrome were found to have three chromosomes (trysomy) containing the Cu,Zn-SOD loci and an increase by 50% of SOD quantity.

Although an increase in SOD activity was found in the trees which are triploid or tetraploid in the experiments performed by the present inventors, correlation between the increase in chromosome number and the increase in SOD level was not confirmed. There may be other reasons for the observed increase in SOD activity in the multiploid plants. However it is concluded that the locus of SOD gene was not found to increase in the individual sample tested.

Rice leaves are known to be ultraviolet light resistant., Its SOD activity was found to be not only higher than those of Japanese cedar, black pine and plus tree of Japanese cypress, but also showed a higher GHS-Px activity than the other trees. Furthermore, catalase activity was hardly detectable in the trees, whereas rice leaves showed a detectable level.

Namely, with an ability that a strong enzyme activity possessed by the tree in response to the environmental oxygen radical and ultraviolet rays, the monocotyledonous plants can acquire the high adaptability against

environmental ultraviolet rays. For example, a fern is a more primitive plant than gymnosperms and angiosperms, and it is well known that a fern can adapt by multiplying their chromosome number to multiploid. Improving adaptability against the environmental challenges by acquiring multiploid has also been reported recently in bacteria.

Furthermore, from the experiments described above, Japanese cedar and Japanese cypress possessing ultraviolet-resistant properties were proved to have triploid and tetraploid of chromosomes. Ultraviolet-sensitive gymnosperms, strengthen their SOD activity by multiplying their ploid of chromosome to protect themselves in response to the increased exposure time to ultraviolet rays and oxygen radical. The adaptability of these species appears to be considerable.

On the other hand, it is considerable that UV-resistant black pine tree increases SOD to protect itself without the relationship of multiploid of chromosome in contrast to the ordinary black pine tree. It is also thought that the UV-resistant black pine trees do not multiplying its chromosome into multiploid. It is generally thought that the increased UVB (the B region of ultraviolet rays is between 280 nm-320 nm) radiation is not connected to oxidation damage with respect to plants, etc. Thus, the case of black pine tree is corresponding to this hypothesis.

There two ways of acquiring multiploid of chromosome by plants: one way is by natural or artificial cross-breeding and the other way is by natural mutation such as branch change or by artificial mutation with colchicines treatment.

Osmunda is a fern. As compared to gymnosperms and angiosperms plants, it is known that many kinds of fern are multiploid. It is well known that monocotyledonous plants such as rice etc. possess greater ultraviolet resistance than black pine, Japanese cedars and Japanese cypress. The experiments performed by the present inventors indicate that both osmunda

and the rice leaves showed a stronger SOD activity than other trees. Gymnosperm plants such as black pine, Japanese cedars, and Japanese cypress, etc., should to demonstrate their actions in response to the real environment, multiplying their chromosome having the SOD gene, as observed by the present inventors observed.

From a survey of references on the agriculture and gardening field, it is confirmed that in the field of improvement of kind of plants such as green tea, apple, banana, tulip, orchid etc, polyploid is used in many cases. But in the conventional Japanese cedar, Japanese cypress, etc, the multiploid species are found to not to grow as well as the ordinary diploid species. This value of multiploid species was utterly not noticed by the forestry industries.

In the plus tree of Japanese cedars and Japanese cypress, the present inventors discovered natural triploid species among the sterile species through cell biological research. The survey reports together with the results obtained by present inventors, reveal that only 41 clones of Japanese cedars and two clones of plus tree of Japanese cypress were found to be the triploid species, respectively. A "plus tree" is a kind of tree which shows a good growth character.

The present inventors made an effort to breed the triploid species by using artificial crossing technology. The effort was performed by crossing diploid and tetraploid species in Japanese cedar and Japanese cypress, respectively. The artificial triploid species obtained by artificial crossing, were confirmed to grow better than the diploid species by the present inventors.

Namely, the better growth of artificial crossing triploid species was confirmed than the conventional triploid one. Thus is concluded that it is possible and beneficial to use the multiploid to breed Japanese cedar and Japanese cypress.

Furthermore in the lipid peroxide assay, the leave homogenate of tetraploid containing a high SOD activity, apparently inhibited the lipid

peroxide formation. This evidence may suggest that trees protect themselves from the damage by oxygen radicals introduced by ultraviolet rays by multiplying their chromosomal number.

Those Japanese cedars except tetraploid cedar, containing comparatively small amount of resin as compared to the other trees, showed a low lipid peroxide level and low SOD activity. Such results were identified in Tables 2-4. Because Japanese cedar contains small amount of resin, it is clear that the formed lipid peroxide can be estimated to be small. Accordingly low SOD activity is sufficient for the treatment of the low levels of lipid peroxide formed.

Namely, it is considered that Japanese cedar do not need to use high SOD activity to treat the low lipid peroxide, because cedar tree does not produce the quantity of lipid peroxide.

According to the research of present inventors, it was confirmed that trees show an excellent adaptability against the increase in harmful oxygen radicals occurring due to the recent acceleration in ozonosphere damage. A fern, an evolutionary more primitive plant than gymnosperms and angiosperms plants, acquired multiploid as its adaptation in response to environment challenges, by multiplying chromosome number.

Also in those needle-leaved plants such as Japanese cedar and Japanese cypress, it is found that their seedlings acquired the ultraviolet resistant property by multiplying their chromosome number as demonstrated in the comparison of the ultraviolet resistant species with the ultraviolet non-resistant species. In particular, triploid and tetraploid trees were found to have this tendency.

On the other hand, although some species resulting from natural cross breeding grew poorly or became sterile, it is confirmed that some species obtain agood growth characteristics by artificial cross breeding.

Namely, for Japanese cedar, Japanese cypress, etc. were found to be

adaptable by multiplying ploid into multiploid. This production of multiploid descendants by artificial crossing etc. can be said as an effective method to protect trees from increased levels of oxygen radicals induced by ultraviolet rays.

This invention is not limited to the form of the above examples. All variations falling within the object of this invention is considered to be encompassed by the invention.

For example, the rational for acquired UV resistant in needle-leaved trees such as Japanese cedar and Japanese cypress was explained and this rational is believed to also apply to broad-leafed trees, evergreen trees, and deciduous trees. On the other hand, while the evidence suggests that trees acquire ultraviolet rays resistance by multiplying their chromosome number, this mechanism for acquiring UV resistance was not confirmed in Japanese black pine trees. There might of course be some species of Japanese black pine trees which acquire the ultraviolet rays resistance by multiploid means, because there are many kinds of pine trees.

It is considered that the strong plant species having ultraviolet resistance can be selected from plant species with the standard ploid of chromosome. And if the selected plants are planted in an area with plenty of sunshine area, the plants will retain strong ultraviolet ray resistance. This will allow for the positive protection of plant ecology and environment in the area.

Though the above explanation is focused on the triploid and tetraploid trees, those multiploid trees such as pentaploid and hexaploid chromosomes will yield similar results.

In the artificially planted forest of the present invention, UV induced damage such as tree death is significantly inhibited. Accordingly, if the scale of ultraviolet radiation on the earth increases due to the progressive destruction of the ozonosphere, the artificially planted forest of the present invention will be reliably sustained. By employing the method of planting

trees according to the present invention, an artificially planted forest having such ultraviolet resistance can be formed.

What is claimed:

1. A method of planting trees, comprising the steps of:

selecting conifer seedlings having a tetraploid chromosome number;

determining which of the selected conifer seedlings exhibit resistance to ultraviolet rays; and

planting in a forest only the selected seedlings which exhibit resistance to ultraviolet rays.

2. The method of planting trees according to claim 1, wherein the conifer seedlings are cedar or cypress.

3. The method of planting trees according to claim 1, further comprising the step of obtaining the chromosome number of the seedlings by increasing the basic number of chromosomes.

4. The method of planting trees according to claim 3, wherein the basic number of chromosomes is increased by mutation.

5. The method of planting trees according to claim 3, wherein the basic number of chromosomes is increased by breeding.

6. The method of planting trees according to claim 1, wherein the step of determining which of the selected conifer seedlings exhibit resistance to ultraviolet rays involves analyzing activity of an anti-oxidation agent in leaves of the seedlings.

7. The method of planting trees according to claim 1, wherein the step of determining which of the selected conifer seedlings exhibit resistance to ultraviolet rays involves analyzing activity of SOD in leaves of the seedlings.

8. A method of growing plants, comprising the step of:

artificially forming vegetation which is significantly resistant to ultraviolet rays, as compared with vegetation constituted of diploid plants, by selectively growing plants whose chromosone number is tetraploid and thus exceeds the basic number of the species and which exhibit resistance to ultraviolet rays.

9. The method of growing plants according to claim 1, further comprising the step of determining which of the plants exhibit resistance to ultraviolet rays.

10. The method of growing plants according to claim 9, wherein the step of determining which of the plants exhibit resistance to ultraviolet rays involves analyzing activity of an anti-oxidation agent in leaves of the plants.

11. The method of planting trees according to claim 9, wherein the step of determining which of the plants exhibit resistance to ultraviolet rays involves analyzing activity of SOD in leaves of the plants.

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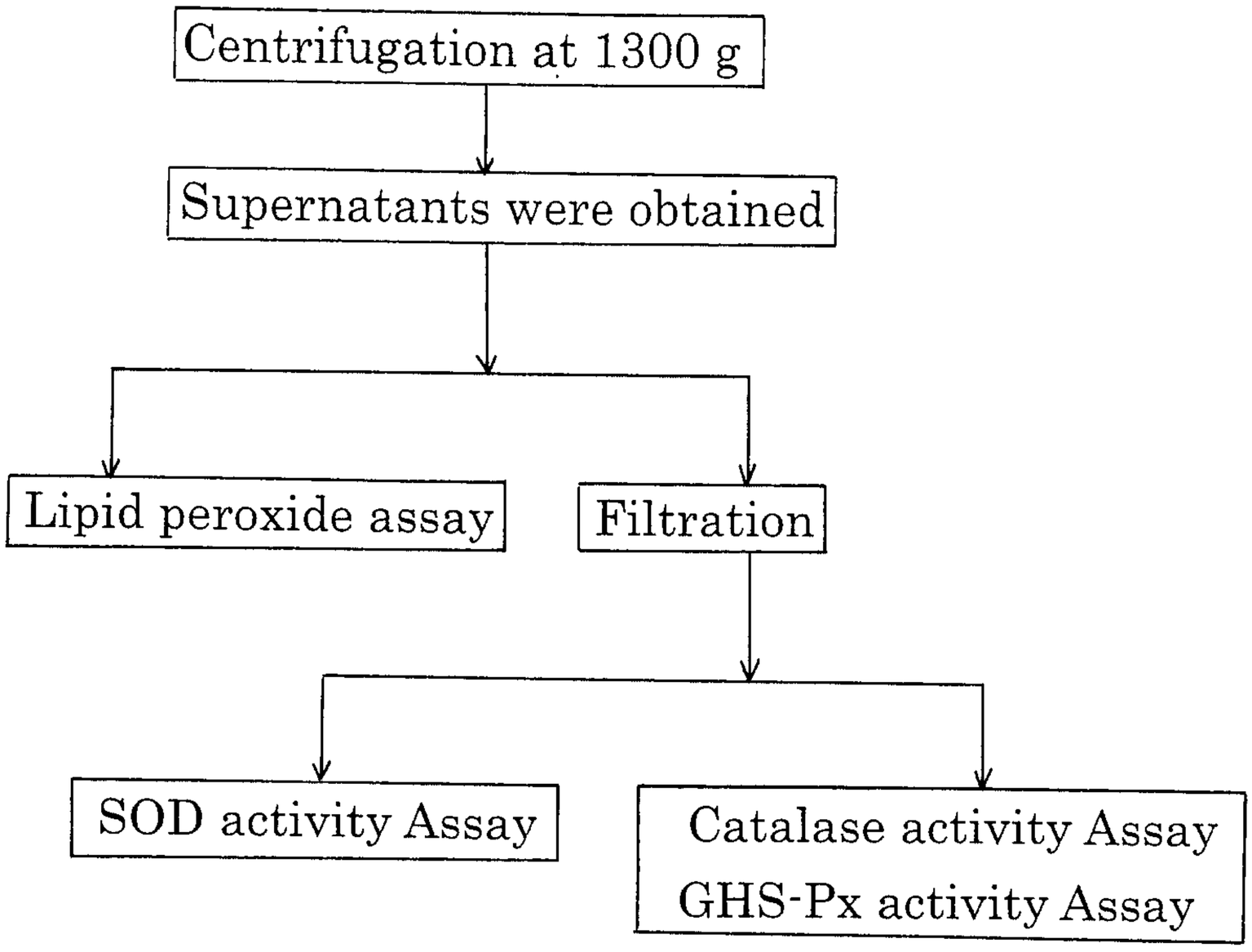
*Fig. 1*


Centrifugation at 1300 g
Supernatants were obtained
Lipid peroxide assay
Filtration
SOD activity Assay
Catalase activity Assay
GHS-Px activity Assay

Centrifugation at 1300 g

Supernatants were obtained

Lipid peroxide assay

Filtration

SOD activity Assay

Catalase activity Assay

GHS-Px activity Assay