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(54) Title: RESISTANCE TO PHYSIOLOGICAL DISORDERS IN LETTUCE

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The invention relates to a method for screening a population of plants for the presence therein of individuals that show a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting and Yellowing, as compared to a control plant, wherein a population of seeds is germinated in darkness and in the presence of ethylene to obtain seedlings that, when having a longer hypocotyl as compared to the original ethylene- sensitive control under ethylene are selected as plants showing a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing. The invention also relates to the plants thus selected.

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RESISTANCE TO PHYSIOLOGICAL DISORDERS IN LETTUCE

Field of the invention

The invention relates to a method for screening a population of plants for the presence therein of individuals, which are altered with respect to their mode of ethylene response. The invention further relates to plants and plant parts, in particular leafy vegetables, thus identified. More in particular, this invention relates to lettuce (*Lactuca sativa* L.) that shows an altered response to ethylene which leads to a reduced susceptibility of this crop species to physiological disorders such as Russet Spotting and Yellowing. The invention also relates to seeds and progeny of these plants and plant parts.

Background of the invention

Breeding of leafy vegetables like lettuce aims at the production of commercial varieties optimally adapted to produce marketable products. Many characteristics need to be taken into account during selection which relate to both input and output traits. One of the most important traits in this respect relates to post-harvest quality, in particular to shelf life. The avoidance of physiological disorders and more in particular Russet Spotting and Yellowing are important elements that can extend the shelf life of a lettuce crop or parts thereof.

Ethylene is a plant hormone generally known to stimulate physiological processes related to senescence. In lettuce this stimulation becomes apparent through the formation of symptoms such as Russet Spotting and Yellowing.

The Russet Spotting disorder is characterised by the appearance of brown spots alongside the midrib of the leaves whereas Yellowing is the general bleaching of leaves which

occurs during senescence as a consequence of chlorophyll breakdown.

Although mature heads of lettuce are known to produce only minute amounts of ethylene, the plants are highly sensitive towards this plant hormone. Therefore, physiological disorders associated with ethylene sensitivity which reduce the post-harvest quality of lettuce are mainly caused by external sources of ethylene. Exposure to such external sources can occur during harvesting, processing and storage of the produce.

For example, when lettuce is transported or stored in the vicinity of ethylene producing fruits such as apples, pears or peaches severe deterioration may occur. Furthermore, when lettuce is processed and used in packaged fresh-cut mixtures there may be limitations with respect to the ingredients which can be used due to ethylene release by one or more of the ingredients.

Russet Spotting is a physiological disorder which is manifested by the appearance of numerous brown spots along the midrib of the leaf. The browning symptoms can spread all over the leaf during the progressive stages of the disorder. Russet Spotting is known to occur especially when mature lettuce heads are stored at lower temperatures (5°C) in the presence of low concentrations (ppm levels) of ethylene.

Symptom formation can be antagonised by applying the plant hormone auxin or calcium. Furthermore, modified atmospheres containing low oxygen levels reduce the speed at which symptoms develop.

At the biochemical level Russet Spotting appears to develop as a consequence of a local stimulation of lignin biosynthesis which causes lignification and cell wall thickening around the area of the leaf where the visual symptoms will appear.

The brown discolouration is caused by the stimulation of phenolic metabolism. The enzyme phenylalanine ammonia lyase (PAL), which has been shown to be induced by ethylene, catalyses the first committed step of the phenylpropanoid pathway. Phenolic compounds which are formed mainly include caffeic acid derivatives as well as a number of flavonoids such as (+)catechin and (-)epicatechin. Subsequent oxidation of these compounds by polyphenol oxidase (PPO) leads to the brown discolouration typically observed in Russet Spotting. Finally, the symptoms may become more severe due to collapsing of tissue and cell death.

Senescence is a naturally occurring, developmental process at the end of a life cycle of a plant or plant organ during which metabolism is reprogrammed in order to remobilize resources into reproductive structures like seeds. Although senescence is a developmental process caused by endogenous factors like physiological age there are many exogenous factors which can modulate senescence.

Yellowing of leaves being the most visible symptom of senescence is a consequence of chlorophyll breakdown during a relatively late stage of senescence which can be enhanced by ethylene once a leaf is receptive. Well-known other stimulating factors of senescence are wounding, darkness and nutrient deficiency. Although ethylene is the most important plant hormone known to stimulate senescence other hormones like jasmonate may also contribute to this process.

From the moment of harvest of the lettuce crop until the moment of consumption, the produce can be exposed to the different exogenous factors contributing to senescence. These can be wounding during harvesting and processing, darkness and nutrient deficiency during storage and ethylene during processing and storage. These factors strongly stimulate the post-harvest disorders which can become apparent as Russet

Spotting and Yellowing. Although these effects are largely cosmetic the product becomes much less attractive and thereby unmarketable.

In order to counter the deterioration effects, many post-harvest measurements can be taken which reduce these effects. For example, one can store the harvested lettuce at low temperatures to retard senescence. Although this may reduce the rate of Yellowing, Russet Spotting may be enhanced. In addition, logistic measurements may be implemented which reduce the transportation time required from the field to the consumer or which prevents the lettuce to be stored in the vicinity of an ethylene source. Furthermore, chemical treatments may be applied which prevent the post-harvest deterioration although food safety and consumer acceptance obviously become an issue.

Many of the post-harvest measurements are successful to some extent but there is certainly room for improvement. Moreover, costs involved may be substantial which is another reason to explore alternatives which reduce the need to apply post-harvest treatments. Preferably, a genetic solution is found which reduces or eliminates the need to take the expensive, preventive measurements which are currently used to maintain the post-harvest quality at a high level.

Summary of the invention

In one aspect, the invention relates to a method for screening a population of plants for the presence therein of individuals that show a reduced susceptibility to ethylene and a physiological disorder that is induced by ethylene as compared to a control plant, which method

comprises: a) providing a population of seeds; b) germinating the seeds in darkness and in the presence of ethylene to obtain seedlings; c) selecting seedlings that show longer hypocotyls than the hypocotyls of an ethylene-sensitive control; d) selfing the selected seedlings to produce seeds; e) germinating one part of the seeds produced from each selected seedling in darkness and in the presence of ethylene and another part of the seeds from each selected seedling in darkness in air; and f) measuring the relative growth of the hypocotyls of the seedlings germinated under ethylene versus the growth of the hypocotyls of the seedlings germinated in air for distinguishing plants that have a longer hypocotyl as compared to the original ethylene-sensitive control both in ethylene and in air from plants that have a longer hypocotyl as compared to the original ethylene-sensitive control only under ethylene, wherein plants that have a longer hypocotyl as compared to the original ethylene-sensitive control only under ethylene are identified as plants showing the reduced susceptibility to ethylene and the physiological disorder.

In another aspect, the invention relates to a use of a lettuce plant of the species *Lactuca sativa* showing a reduced susceptibility to ethylene and a physiological disorder that is induced by ethylene as compared to a control plant, seed of which plant is deposited with the NCIMB on 3 January 2007 under accession number NCIMB 41449, NCIMB 41450, NCIMB 41448, NCIMB 41451, NCIMB 41452 or NCIMB 41453, as a crop.

In another aspect, the invention relates to a use of a lettuce plant as defined above as a source of seed.

In another aspect, the invention relates to a use of a lettuce plant as defined above or a part thereof as a source of propagating material.

In another aspect, the invention relates to a use of a lettuce plant as defined above or a part thereof for consumption.

In another aspect, the invention relates to a use of a lettuce plant as defined above for conferring the trait of the reduced susceptibility to ethylene and the physiological disorder that is induced by ethylene on another lettuce plant.

In another aspect, the invention relates to a use of seeds of which a representative sample was deposited under accession numbers NCIMB 41449, NCIMB 41450, NCIMB 41448, NCIMB 41451, NCIMB 41452 and NCIMB 41453 for transferring the trait of reduced susceptibility to ethylene and a physiological disorder that is induced by ethylene into another agronomically valuable lettuce plant of the species *Lactuca sativa*.

In another aspect, the invention relates to a use of seeds as defined above to produce a vegetable product, which vegetable product is lettuce.

Detailed description of the invention

It is the object of the present invention to provide a screening method which allows to identify ethylene-insensitive plants. It is a further object of the invention to provide plants that are obtainable by the method.

This object is achieved according to the invention by a method for screening a population of plants for the

presence therein of individuals that show a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting and Yellowing, as compared to a control plant, which method comprises:

a) providing a population of seeds;

b) germinating the seeds in darkness and in the presence of ethylene to obtain seedlings;

c) selecting seedlings that show longer hypocotyls than the hypocotyls of an ethylene-sensitive control;

d) selfing the selected seedlings to produce seeds;

e) germinating one part of the seeds produced from each selected seedling in darkness and in the presence of ethylene and another part of the seeds from each selected seedling in darkness in air; and

f) measuring the relative growth of the hypocotyls of the seedlings germinated under ethylene versus the growth of the hypocotyls of the seedlings germinated in air for distinguishing plants that have a longer hypocotyl as compared to the original ethylene-sensitive control both in ethylene and in air from plants that have a longer hypocotyl as compared to the original ethylene-sensitive control only under ethylene,

wherein plants that have a longer hypocotyl as compared to the original ethylene-sensitive control only under ethylene are identified as plants showing a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing.

The invention is based on the hypothesis that ethylene-insensitive plants, in particular lettuce, would be resistant to post-harvest physiological disorders such as Russet Spotting and Yellowing.

In a preferred embodiment, the method of the invention comprises the further step of testing plants that have been identified in the previous steps as showing a reduced susceptibility to ethylene for their resistance to Russet spotting and/or Yellowing.

Hypocotyl length and root length can be observed by comparison to the standard and scoring from 1, which means equal to the ethylene-sensitive standard variety under ethylene, to 3, which means equal to the ethylene-sensitive standard variety under air. If desired, measurements in millimetres can be made. Using one of these measurements, simple statistical analyses like a t-test, well-known to the person skilled in the art, can be performed to establish whether a plant or group of plants is significantly less sensitive to ethylene than the ethylene-sensitive standard, like cv. 'Troubadour' (Rijk Zwaan, De Lier, NL). The applied significance level of a one-sided test is 0.001.

For the mutants, the statistical comparison is preferably made between the hypocotyl lengths and optionally the root lengths of the original variety that was used for the mutagenesis treatment, which is the best available standard, and the hypocotyl and root lengths of the individual mutants and/or their offspring.

For finding ethylene-insensitive plants in existing plant material, one or more representative samples of varieties, breeding lines and/or gene bank accessions are selected. The statistical comparison is then suitably made between the hypocotyl and root lengths of the individual accession under investigation and the rest of the population. When statistically testing individuals for significantly longer hypocotyls and/or roots multiple comparison tests may be needed to maintain proper overall significance levels, for example Dunnett's multiple comparison test with one standard (Dunnett, CW, J. Amer. Statist. Assoc. 50:1096-1121 (1955)).

The plants to be screened are usually leafy vegetable plants, in particular plants belonging to the genus *Lactuca* and in particular to the species *Lactuca sativa*.

The plant population is preferably a population of mutant plants because the chances are higher to find a plant of the invention in such variant population. However, any other population of plants that differ in their genetic constitution may be screened according to the invention.

The population of mutant plants is preferably obtained by a mutagenesis treatment using chemicals and/or irradiation. Mutagenesis treatments are well-known and will be further described hereinbelow.

The concentration of ethylene in step b) is at least 10 µg/litre, preferably between 11 and 25 µg/litre. The concentration of ethylene in step d) is about 4 to 5 µg/litre.

The selection step in the screening method of the invention is based on the elongation of the hypocotyls. Exposure of dark-grown germinating seedlings to ethylene causes radial swelling of the hypocotyl and inhibition of root and hypocotyl growth. This phenomenon is generally referred to as the triple response (see for example Guzman, P & Ecker, J, Plant Cell 2:513-523 (1990)). The reproducibility of this response allows to screen for mutants that show an altered triple response in the presence or absence of ethylene. In addition to or instead of measuring the elongation of the hypocotyls, the selection according to the invention may be based on one or more of the other elements of the triple response test.

According to a further aspect thereof, the invention provides plants showing a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting and Yellowing, as compared to a control plant, which plants are obtainable by subjecting a population of plant seeds to a screening method of the invention and selecting plants from the population that show longer hypocotyls in comparison with

an ethylene-sensitive control as plants showing a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing.

The plant of the invention is preferably a leafy vegetable plant, in particular a plant which belongs to the genus *Lactuca* and in particular to the species *Lactuca sativa*.

With the screening method of the invention, ethylene-insensitive mutants were identified and selected. Seeds of these mutants were deposited with NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA UK on 3 January 2007 and have been given the accession numbers as listed in **Table 1**. Details about seed descendance of the deposits are given in Example 3 and in Example 4. These deposits are made because they have the single specific characteristic of significantly reduced susceptibility to ethylene. They were not tested for DUS-criteria for variety registration, i.e. distinguishability, uniformity, stability on all registration characteristics, and are not expected to meet these criteria in any way.

Table 1

Plant no.	Internal reference (=NCIMB reference)	NCIMB accession number
00D.7856	07D.826509	41449
00D.6876	07D.826514	41450
00D.7871	07D.826502	41448
00D.6883	07D.826522	41451
00D.6896	07D.826540	41452
00D.7845	07D.826542	41453

The invention further relates to plants that show a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing and are obtainable by crossing a plant of the invention with another plant of the same species. The feature "reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing" can thus be brought into other plants that originally do not have the feature. Whether the plants resulting from such a cross are indeed plants of the invention can be tested by subjecting these plants to the screening method of the invention.

The invention further relates to progeny of a parent plant of the invention that shows the reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing. Such progeny may be many generations removed from the parent. Provided that the feature "reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing" is present, the progeny plant is a plant of the invention.

The invention further relates to parts of the plants of the invention. The plant parts are for example lettuce heads or leaves, such as baby leaves, processed heads or cut leaves.

Plant parts of the invention can be used in tissue culture to regenerate plants that have the reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing as found in the plant from which the tissue for the tissue culture is derived. Such regenerated plants are also part of this invention.

The invention further relates to seed of a plant of the invention. From the seed plants can be grown that also have the feature "reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or

Yellowing". Whether or not the seeds and thus the plants grown therefrom have retained that feature can be tested in the screening method of the invention. The invention also relates to further generation seeds that retain the reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing as found in the original seeds.

According to a further aspect thereof the invention relates to a vegetable product, comprising a plant or part thereof of the invention. Preferably, the vegetable is a leafy vegetable, more in particular the vegetable is lettuce.

When screened in the method of the invention, the vegetable product shows reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing.

It should be noted that the screening method of the invention is a relatively simple method. Plants of the invention that have a reduced susceptibility to ethylene and physiological disorders, in particular Russet Spotting or Yellowing can be identified in any variant population that is sufficiently large. It does not require undue experimentation to reproduce the invention and the plants of the invention must therefore not be construed as to be limited to the ones that are deposited.

As a result of their ethylene insensitivity, the lettuce plant material and seed derived therefrom of the invention show a strong increase with respect to their post-harvest quality especially related to the senescence associated phenomena such as Russet Spotting and Yellowing.

Detailed description of the invention

As ethylene is the most important plant hormone in stimulating senescence and as Russet Spotting and Yellowing

are associated with senescence, a genetic approach was taken in which genetic variants were produced and selected for ethylene insensitivity. It was found that this indirectly leads to the identification of mutants which are affected in their post-harvest senescence response.

Detailed studies using plant model species such as *Arabidopsis thaliana* as well as crop species have provided information on the biochemical pathways involved in the biosynthesis and perception of ethylene. Many allelic forms of genes have been characterised for their role in this respect providing an intricate picture of ethylene function in plants. Ethylene, like other plant hormones, plays a important role in many physiological processes embedded in complicated interactive regulatory networks.

The spatial and temporal activity of the hormone is *inter alia* determined at the level of gene expression underlying ethylene biosynthesis, hormone perception, signal transduction and activation of downstream effector proteins.

Allelic variants of genes involved at different levels can determine the strength of the response as well as the level of cross-talk to other signalling pathways. As ethylene biosynthesis and perception are poorly characterised in lettuce and in order to allow the identification of different response strengths and their underlying allelic variants it was reasoned that an unbiased approach may be more successful in this respect as compared to a targeted gene modification approach.

Such unbiased approach encompasses preferably a chemical or physical random mutagenesis procedure combined with an efficient phenotypic screening and selection procedure based on a response of etiolated seedlings to ethylene. Such seedling-based selection system is by far more efficient in terms of numbers of plants which can be assessed

per man hour as compared to the use of mature lettuce heads. Furthermore, the time to produce plant material for screening is obviously much more reduced in the case of seedlings as compared to mature heads.

A further advantage of this approach is the use of the selection conditions at the seedling stage as predictive phenotypic marker for the post-harvest trait in consecutive generations once a successful event has been identified. A clear risk exists in the fact that selection at the seedling stage may not lead to genetic variants which express the selected trait at the mature, post-harvest level. Furthermore it is recognised that plant hormones like ethylene are involved in many physiological processes which may lead to pleitotropic effects. These can either be positive or negative depending on the crop and its cultivation conditions. The approach taken therefore preferably comprises the following steps:

1. Generation of a mutant population by treatment of seeds or plant tissues with mutagenic agents like ethane methylsulfonate (ems) or gamma-irradiation.

2. The set-up of an efficient phenotypic screen in which selection is based on a response of etiolated seedlings of plants, in particular lettuce, to ethylene which is characterised by a strong reduction of the hypocotyl elongation, a shortened, thickened root and an exaggerated apical hook curvature. This response is called the triple response which is typical for etiolated seedlings and found to occur to various degrees in many plant species when exposed to atmospheres containing ethylene (see for example Ecker J, Science 268(5211):667-675 (1995)).

3. Characterisation of the mutants modified in at least one test of their triple response with respect to post-harvest deterioration which comprise Russet Spotting and

Yellowing. Optionally, determination of pleiotropic effects of the modification to exclude negative pleiotropic effects that would affect the value of the plants.

In the screening method of the invention the population of plant seeds to be tested is thus preferably a population of mutant seeds that can be obtained by a method comprising:

a) treating M0 seeds of a plant species to be modified with a mutagenic agent to obtain M1 seeds;

b) growing plants from the thus obtained M1 seeds to obtain M1 plants;

c) producing M2 seeds by self-fertilisation of M1 plant; and

d) optionally repeating step b) and c) n times to obtain M1+n seeds.

The M1+n seeds thus obtained are then germinated in darkness and in the presence of high ethylene concentration to obtain seedlings. Subsequently, seedlings are selected that do not show a response to ethylene. The response to be measured is the development of hypocotyls that are elongated as compared to the hypocotyls of an ethylene-sensitive seedling.

Then, progeny of each selected plant is split up and grown in the dark: half of the progeny under ethylene, half of the progeny under air. Relative growth of the hypocotyl under ethylene versus air is measured for each progeny under both conditions. These observations are used to distinguish progenies which have a longer hypocotyl than the original ethylene-sensitive control under ethylene, but also under air, and which can therefore be concluded to be ethylene-sensitive and not desired, from the progenies truly have a reduced susceptibility to ethylene.

An example of a well-known mutagen is ems. Ems alkylates primarily G residues of a DNA strand which during DNA replication causes pairing with T in stead of C. Therefore, GC basepairs change to AT basepairs at a frequency which is determined by the effective dose of ems and the activity of the mismatch repair system of the plant. The effective dose of ems depends on the concentration used, the seed size and other physical properties and the time of incubation of the seeds in the ems solution. The seeds which have been treated with ems are typically called M1 seeds. As a consequence of the treatment, the tissues of the M1 seeds contain random point mutations in the genomes of their cells and those present in the subpopulation of cells which will form the germline tissue (germinal cells) will be transferred to the next generation which is called M2. Mutations or combinations thereof which are haplo-insufficient thereby causing sterility or which induce embryo lethality will not be transferred to the M2 generation.

A similar procedure as described above for the use of ems applies for other mutagenic agents as well. The M2 population can be used in screening procedures aimed at a reduced triple response of etiolated seedlings.

Other mutagenic agents, in particular alkylating mutagenic agents, are diethyl sulfate (des), ethyleneimine (ei), propane sultone, N-methyl-N-nitrosourethane (mnu), N-nitroso-N- methylurea (NMU), N-ethyl-N-nitrosourea (enu), sodium azide.

Alternatively, the mutations are induced by means of irradiation, which is for example selected from x-rays, fast neutrons, UV irradiation.

In another embodiment of the invention the mutations are induced by means of genetic engineering, such as by means of use of chimeric oligonucleotides, homologous

recombination, introduction of modified target genes which compete with the endogenous product, downregulation through RNA interference, etc.

The technology which allows to modify gene targets residing in the genome of a plant in a specific manner is known to the person skilled in the art. For example, chimeric oligonucleotides have been demonstrated to be effective mutagens with a specific mode of action. Another approach is to modify gene targets through homologous recombination or gene targeting. Using such approach, a fragment of a gene is exchanged by an introduced DNA fragment containing a desired modification. Transgenic approaches are also feasible in which modified target genes are introduced which compete with the endogenous product. This may lead to dominant negative effects. Moreover specific downregulation of the expression of genes is feasible through RNA interference.

In case mutagenic oligonucleotides, gene targeting or transgenic approaches are used to modify a genetic factor involved in ethylene function, obviously, the primary structure of the relevant genes should be known. Currently however, for lettuce knowledge on such genes is limited.

Preferably, the invention further comprises pyramiding alleles of reduced susceptibility towards ethylene.

Production of M1 and M1+n seeds is suitably effected by means of self-pollination.

The invention further relates to plants or plant parts, which have in their genome genetic information which is responsible for the reduced susceptibility towards physiological disorders such as Russet Spotting and Yellowing and is found in the genome of a lettuce plant as listed in **Table 1**.

Progeny of the plants as claimed are also part of this invention. "Progeny" as used herein is intended to encompass all plants having the same or a similar reduced susceptibility towards ethylene and physiological disorders, in particular Russet Spotting or Yellowing, as the original plants described herein and being derived therefrom in any way, such as by sexual reproduction, like self-fertilisation or cross-fertilisation with another plant of the same genus, or vegetative reproduction such as cutting, tissue culture, haploid culture, protoplast culture, protoplast fusion or other techniques. Such progeny is thus the first generation of plants as identified according to the invention, as well as the first generation of plants derived by one or more of these techniques, but also every further generation of plants derived by one or more of these techniques, provided that the derived plants have the reduced susceptibility.

In order to determine the response of the etiolated lettuce seedlings towards ethylene, use was made of an especially designed plastic containers in which lettuce seedlings were grown on filter papers under an atmosphere in which ethylene levels can be varied. It was indeed found that the lettuce seedlings responded to the presence of ethylene by a reduced elongation of the hypocotyl which in principle would allow to select for ethylene-insensitive mutants in case such mutants reside in the available population and in case the insensitivity is expressed phenotypically at the seedling level under the experimental conditions which were applied.

By growing large numbers of etiolated lettuce seedlings from a population containing randomly induced mutations under an ethylene containing atmosphere, it was found that seedlings showing reduced ethylene sensitivity as compared to the ethylene sensitivity of the starting

population can be obtained and selected. Seedlings were selected showing hypocotyl elongation to an extent comparable to a situation in which the atmosphere was composed of air without ethylene. The seedlings identified in this manner were qualified as being insensitive to ethylene.

In order to confirm that the ethylene-insensitive mutants are resistant to Russet spot and Yellowing the mutants identified in the screen are tested for their resistance to Russet spot and/or Yellowing.

A Russet spot test suitably comprises storing harvested mature heads in a closed container at a temperature of 8°C in the dark, and exposing them to ethylene gas at a concentration of between 6 and 7 vpm (volume parts per million) and assessing the presence of symptoms of Russet spot after 7 days, preferably after 9 days. Suitably an ethylene-sensitive control head is incubated together with the plants to be tested and Russet spotting of the plants to be tested is compared therewith.

A Yellowing test comprises storing mature heads of the lettuce plants to be tested in an ethylene-free storage chamber at 8°C and assessing the yellowing of the base leaves after 10 days, preferably after 14 days. Yellowing resistant plants are plants that do not show yellowing of the base leaves after 14 days. Suitably an ethylene-sensitive control head is incubated together with the plants to be tested and the Yellowing response of the plants to be tested is compared therewith.

As illustrated in the Examples, it has been demonstrated for the first time that ethylene-insensitivity leads to resistance to Russet spotting and to resistance to Yellowing that is not induced by ethylene.

Surprisingly, seedlings which showed an elongation response which was reduced as compared to the control

conditions but which were longer as compared to the sensitive controls were also found which were considered to be insensitive to ethylene as well albeit partial.

The variable levels of ethylene insensitivity which were observed may reflect either the presence of different mutant loci or different allelic forms of identical loci affecting this trait in the original population.

In case of recessive mutation these two possibilities can easily distinguished by carrying out allelism assays which comprises the crossing of the two mutant events and determining the phenotype of the hybrid. In case of allelism of the mutations, the ethylene insensitivity will be apparent in the F1 whereas in case the phenotype in the mutants is determined by different recessive loci this will not be the case.

As random mutagenesis was applied as a preferred *means to generate the starting population, mutations in the* genetic background may also contribute to the variation of the seedling phenotype under the experimental conditions. In order to discriminate between single mutations of different strength and a combined effect of mutation in the genetic background, backcrosses should be performed to create uniform genetic backgrounds for the different ethylene insensitive events. Such procedure is further relevant in order to determine whether mutations at specific loci involved in ethylene sensitivity display pleiotropic effects.

The M2 plants thus selected on the basis of a reduced response to ethylene were used to grow M3 seeds. In some cases M3-plants grown from the M3-seeds were selected for reduced ethylene sensitivity in a triple response test and selfed to produce M4-seeds. This was even repeated until M5 in a few cases.

In one case an M3-plant grown from M3-seed was selected for reduced ethylene sensitivity in a triple response test, and crossed with an ethylene sensitive and Russet spot sensitive parent to obtain F1-seed. An F1-plant grown from the F1-seed was selfed to produce F2-seeds. An F2-plant grown from these F2-seeds was selected for reduced ethylene sensitivity in a triple response test, and selfed to produce F3-seeds. This F3-line was added to the set of M3 and M4 inbred lines Subsequently, the inbred lines descending from the ethylene insensitive events were re-evaluated for their response to ethylene. The level of insensitivity of each inbred line was scored on the basis of the relative growth of the seedling under an ethylene containing atmosphere versus air. Based on this criterion 12/54 lines which were scored ethylene insensitive earlier were now classified as being sensitive. These false positives during the initial screen at the M2 level, can readily be eliminated during the re-evaluation of the events in the next generation.

The inbred lines that showed a confirmed and significant ethylene-insensitivity in the ethylene test were resown and grown in a greenhouse under regular lettuce production conditions to produce mature heads that were assessed for Russet Spotting and Yellowing (see also the Examples).

As negative controls ethylene-sensitive plants were grown which originate from the population which was used to select the ethylene-insensitive events. Surprisingly, the seeds of all ethylene-insensitive mutants germinated normally i.e. comparable to the seeds of a near-isogenic ethyleen-sensitive control plant when planted in potting soil.

This contrasts sharply to the situation in other plant species such as *Arabidopsis thaliana* which show a

strong reduction in germination capacity when planted in potting soil (Harpham, N.J.V. et al (1991) Annals of Botany 68, 55-61). Apparently for lettuce it is possible to grow ethylene-insensitive mutants according to a normal cultivation practice which in many cases includes sowing in potting soil blocks or potting soil plugs.

After cultivation, mature heads were harvested and exposed to ethylene. One week of post-harvest incubation of heads at 8°C under an ethylene containing atmosphere resulted in a strong induction of Russet Spotting of the heads of the ethylene-sensitive control plants. However, 29 out of 37 ethylene-insensitive events which were assessed showed no sign of Russet Spotting at all which surprisingly demonstrates that ethylene resistance which was selected for at the seedling level can reduce physiological disorders at the mature plant level, even at the post-harvest stage.

Yellowing resistance could be demonstrated for a number of ethylene-insensitive events under ethylene-free storage conditions at sub-optimal temperature. This is surprising, because until now Yellowing resistance has only been reported in the presence of ethylene (Saltveit et al., Postharvest Biology and Technology 27: 277-283 (2003)).

The present invention will be illustrated in the Examples that follow and that are not intended to limit the invention in any way. More in particular, the experiments in the Examples are performed with lettuce but the invention is more broadly applicable to other plant species that encounter similar post-harvest difficulties when contacted with ethylene.

In the examples reference is made to the following figure.

Figure: Phenotype of mature heads of lettuce after exposure to ethylene at 8°C in the dark during 9 days. The

left panel shows a representative sample of ethylene-resistant (Ethylene R) and ethylene-sensitive control (- control) heads of the variety Troubadour. The right panel shows a similar picture of the mutants derived from the variety Apache.

EXAMPLES

EXAMPLE 1

Genetic modification of lettuce by ethyl methane sulfonate (ems)

Seeds of the lettuce varieties Troubadour, Apache and Yorvik (all three from Rijk Zwaan, De Lier, the Netherlands)were treated with ems by submergence of approximately 2000 seeds per variety into an aerated solution of either 0.05% (w/v) or 0.07% (w/v) ems during 24 hours at room temperature.

Approximately 1500 treated seeds per variety per ems dose were germinated and the resulting plants were grown in a greenhouse in the Netherlands from May to September to produce seeds.

After maturation, M2 seeds were harvested and bulked in one pool per variety per treatment. The resulting 6 pools of M2 seeds were used as starting material to identify the individual M2 plants containing reduced susceptibility alleles.

The efficacy of the genetic modification procedure was assessed by determining the occurrence of bleached plants, which is indicative for chlorophyll loss due to modifications in genes directly or indirectly involved in the formation or accumulation of chlorophyll. In all 6 pools of M2 seeds individual plants, which are bleached, were observed which demonstrates that the applied treatments result in genetic modifications.

EXAMPLE 2

Identification of lettuce plants which have obtained reduced susceptibility alleles for ethylene

M2 lettuce seeds were germinated on paper in a small plastic container with an ethylene concentration of 10 20 vpm (volume parts per million) at 16°C in dark. 1 vpm contains 0.41 µmol/litre or 1.14 µg/litre. Ethylene-insensitive mutants were compared to ethylene-sensitive controls, and selected on the basis of elongated hypocotyl and/or root (i.e. triple response test). These ethylene-insensitive mutants were grown to produce M3 lines by self fertilisation. These M3 lines were re-tested with the triple response test to confirm ethylene insensitivity.

When a line was segregating for ethylene insensitivity, plants were selected followed by one or two additional cycles of inbreeding, and a final triple response test to select homozygous ethylene-insensitive lines, if possible.

In one case an M3-plant grown from M3-seed was selected for reduced ethylene sensitivity in a triple response test, and crossed with an ethylene sensitive and russet spot sensitive parent Troubadour to obtain F1-seed. An F1-plant grown from the F1-seed was selfed to produce F2-seeds. An F2-plant grown from these F2-seeds was selected for reduced ethylene sensitivity in a triple response test, and selfed to produce F3-seeds. The resulting F3-line was added to the set of 53 M3, M4, and M5 inbred lines. In this case the F3-line was the only representant of the original M2-mutant plant, because there were no selfed seeds left from these plant.

This set of 54 M3 and M4 lines from Example 1 were germinated on peat blocks in a closed container under ethylene concentration between 4 and 4,5 vpm in the dark. Ethylene-insensitive mutants were identified by their longer hypocotyls in comparison with sensitive control varieties (Troubadour, Apache, Yorvik, Sensaï). Results are presented in Table 1. 42 Out of 54 lines that were identified by the triple response test appeared to be at least partial ethylene-insensitive in the ethylene test. These lines *represent 40 M2-plants, because 2 M2-plants were represented* twice.

Table 2 shows the results.

Table 2

Ethylene response of mutant lines in a closed container test and a triple reponse test (trt). Observations on hypocotyl and root are indicated separately, when no consistent response is shown. Intermediate response is indicated as partial. R=ethylene-insensitive; S=ethylene-sensitive; R/S=segregating; T=Troubadour; A=Apache; Y=Yorvik; hypo=hypocotyl

plot	seed no.	origin	ethylene	trt	plot	seed no.	origin	ethylene	trt
1	YORVIK		S	S					
2	TROUBADOUR		S	S	32	01D.85755	T	R/S	R/S
3	APACHE		S	S	33	01D.85762	A	partial R	R hypo; S root
4	00D.88531	Y	S	R/S hypo; R root	34	03D.90452	A	R	R
5	00D.88539	Y	S	S	35	01D.85764	A	partial R/S	R hypo; S root
6	01D.85717	Y	S	R	36	02D.90070	A	R	R
7	01D.85720	Y	S	R	37	03D.90457	A	R	R
8	01D.85732	Y	S	S	38	01D.85768	A	R	R
9	00D.88533	Y	S	S	39	02D.90128	A	R	R
10	00D.88538	Y	S	R	40	02D.90129	A	R	R
11	03D.74008	Y	R	S	41	02D.90130	A	R	R
12	02D.90749	Y	S	S	42	03D.90462	A	R	R

13	00D.88550	Y	S	S
14	01D.85739	Y	S	S
15	00D.88565	T	R	R
16	02D.91445	T	R	R
17	00D.88577	T	R/S	R hypo; S root
18	02D.91446	T	R	R
19	02D.91447	T	R	R
20	01D.85754	T	S?	R/S
21	02D.90047	T	R	R
22	01D.85758	T	R	R
23	00D.88564	T	S	S
24	02D.91442	T	R	R
25	00D.88569	T	R	R
26	00D.88573	T	R	R
27	00D.88578	T	R	R
28	00D.88582	T	R	R
29	01D.85748	T	R/S	R/S
30	01D.85750	T	S	R
43	03D.90464	A	R	R
44	02D.90133	A	R	R
45	02D.90134	A	R	R
46	00D.88600	A	R/S	R
47	02D.91479+	A	R	R
48	01D.85772	A	R	S hypo; R root
49	04D.800957	A	R	S hypo; R root
50	02D.90091	A	R	R
51	04D.800960	A	R	S hypo; R root
52	04D.800963	A	R	R
53	01D.85780	A	R	R
54	01D.85756	T	R	R
55	*02D.90036*	T	*partial R*	S hypo; R root
56	04D.801660	Y	partial R	R/S hypo; S root
58	04D.800900	T	R	S hypo; R root
59	03D.90323	T	R	R
60	Sensai		S	S

EXAMPLE 3

Identification of lettuce plants which have obtained reduced susceptibility alleles for Russet Spot

Thirty-seven ethylene-insensitive lines from the forty-two lines found in Example 2 were sown in a greenhouse to produce mature heads under regular lettuce production conditions (location: Maasdijk, the Netherlands; sowing: 10 January; transplanting: 17 February; harvest: 18 April). The harvested mature heads were stored in a closed container at a temperature of 8°C in the dark. They were exposed to ethylene gas at a concentration of between 6 and 7 vpm (volume parts per million). 1 vpm contains 0.41 µmol/liter or 1.14 µg/liter. After 9 days, plants displaying Russet Spotting were identified. All control plants, except Yorvik displayed Russet Spotting. 29 Out of 37 lines tested showed absence of Russet Spotting or less Russet Spotting as compared to the original variety (**Table 3**).

Six lines were chosen for multiplication and deposition at NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA, UK. The first line is numbered 02D.91445. It is an M4-line descending from the ethylene-insensitive Troubadour-M2-plant 00D.7856. An ethylene-insensitive M4-plant was selected from 02D.91445 in the closed container test in Example 2 and selfed to produce M5-seeds. Sixteen M5-plants were grown from these seeds to produce an M6 seed lot by selfing. This seed lot is numbered 07D.826509 and it is deposited as NCIMB-number 41449 (first line).

The second line is numbered 02D.90047. It is an M4-line descending from the ethylene-insensitive Troubadour-M2-plant 00D.6876. An ethylene-insensitive M4-plant was selected from 02D.90047 in the closed container test in Example 2 and selfed to produce M5-seeds. Sixteen

M5-plants were grown from these seeds to produce an M6 seed lot by selfing. This seed lot is numbered 07D.826514 and it is deposited as NCIMB-number 41450 (second line). The absence of russet spotting for this origin was established by testing the M5-line 03D.90323, descending from an M4-plant of 02D.90047.

The third line is numbered 00D.88578. It is an M3-line descending from the ethylene-insensitive Troubadour-M2-plant 00D.7871. Sixteen M3-plants were grown from the 00D.88578 to produce an M4 seed lot by selfing. This seed lot is numbered 07D.826502 and it is deposited as NCIMB-number 41448 (third line).

The fourth line is numbered 03D.90452. It is an M5-line descending from the ethylene-insensitive Apache-M2-plant 00D.6883. An ethylene-insensitive M5-plant was selected from 03D.90542 in the closed container test in Example 2 and selfed to produce M6-seeds. Sixteen M6-plants were grown from these seeds to produce an M7 seed lot by selfing. This seed lot is numbered 07D.826522 and it is deposited as NCIMB-number 41451 (fourth line).

The fifth line is numbered 01D.85780. It is an M3-line descending from the ethylene-insensitive Apache-M2-plant 00D.6896. An ethylene-insensitive M3-plant was selected from 01D.85780 in the closed container test in Example 2 and selfed to produce M4-seeds. 16 M4-plants were grown from these seeds to produce an M5 seed lot by selfing. This seed lot is numbered 07D.826540 and it is deposited as NCIMB-number 41452 (fifth line).

The sixth line is numbered 04D.801660. It is an F3-line descending from a cross between the ethylene-insensitive Yorvik-M3-plant 02D.8484 and a plant of the ethylene sensitive variety Troubadour. The M3-plant 02D.8484 descended from the ethylene-insensitive

Yorvik-M2-plant 00D.7845. An ethylene-insensitive F3-plant was selected from 04D.801660 in the closed container test in Example 2 and selfed to produce F4-seeds. 16 F4-plants were grown from these seeds to produce an F5 seed lot by selfing. This seed lot is numbered 07D.826542 and it is deposited as NCIMB-number 41453 (sixth line).

Fig. 1 shows resistant and control plants.

EXAMPLE 4

Identification of lettuce plants that show less post-harvest yellowing

Ethylene-insensitive lines grown under greenhouse conditions as described for Example 3 were harvested and mature trimmed heads were stored in an ethylene-free storage chamber at 8°C. After two weeks base leaves of the green control varieties Yorvik and Troubadour started to turn yellow. At that moment and even 1 week later three lines appeared to have less yellow base leaves than their origin varieties, which were the controls used in this trial. These lines were numbered 04D.800900, 03D.90323, 04D.801660.

Although a relationship is reported between leaf yellowing and ethylene presence (Saltveit et al. (2003) Postharvest Biology and Technology 27:277 283), it is surprising that even in conditions without ethylene, some of the ethylene-insensitive lines are expressing a less yellowing phenotype.

Table 3

Observations of russet spotting on mature heads after 9 days of storage in ethylene conditions. 0=symptoms absent; 1=weak symptoms; 2=strong symptoms; NA=not available

seed no.	Ethylene	trt	russet spot	seed no.	ethylene	trt	russet spot
01D.85762	partial R	R hypo; S root	2	00D.88565	R	R	0
03D.90452	R	R	0	02D.91445	R	R	0.5
01D.85764	R/S partial R	R hypo; S root	1	00D.88577	R/S	R hypo; S root	0
02D.90070	R	R	0	02D.91446	R	R	0
03D.90457	R	R	0	02D.91447	R	R	0
01D.85768	R	R	0	01D.85754	S?	R/S	0
02D.90128	R	R	2	01D.85756	R	R	0
02D.90129	R	R	2	02D.90047	R	R	NA
02D.90130	R	R	2	01D.85758	R	R	0
03D.90462	R	R	2	02D.91442	R	R	0
Apache	S	S	2	Troubadour	S	S	1
03D.90464	R	R	0	00D.88569	R	R	0
02D.90133	R	R	2	00D.88573	R	R	0
02D.90134	R	R	2	00D.88578	R	R	0
00D.88600	R/S	R	NA	00D.88582	R	R	0

	02D.91479	R	R	0	01D.85748	R/S		R/S	0 1
	01D.85772	R	S hypo; R root	0					
	04D.800957	R	S hypo; R root	0	02D.90036	partial R		S hypo; R root	NA
	02D.90091	R	R	0	01D.85755	R/S		R/S	NA
	04D.800960	R	S hypo; R root	0	04D.800900	R		S hypo; R root	0.5
	04D.800963	R	R	2	03D.90323	R		R	0
	01D.85780	R	R	0	4D.801660	partial R		R/S hypo; S root	0 2
	Apache	S	S	2	Yorvik	S	S		0

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. Method for screening a population of plants for the presence therein of individuals that show a reduced susceptibility to ethylene and a physiological disorder that is induced by ethylene as compared to a control plant, which method comprises:

a) providing a population of seeds;

b) germinating the seeds in darkness and in the presence of ethylene to obtain seedlings;

c) selecting seedlings that show longer hypocotyls than the hypocotyls of an ethylene-sensitive control;

d) selfing the selected seedlings to produce seeds;

e) germinating one part of the seeds produced from each selected seedling in darkness and in the presence of ethylene and another part of the seeds from each selected seedling in darkness in air; and

f) measuring the relative growth of the hypocotyls of the seedlings germinated under ethylene versus the growth of the hypocotyls of the seedlings germinated in air for distinguishing plants that have a longer hypocotyl as compared to the original ethylene-sensitive control both in ethylene and in air from plants that have a longer hypocotyl as compared to the original ethylene-sensitive control only under ethylene,

wherein plants that have a longer hypocotyl as compared to the original ethylene-sensitive control only under ethylene are identified as plants showing the reduced susceptibility to ethylene and the physiological disorder.

2. Method as claimed in claim 1, wherein the physiological disorder is Russet Spotting and/or Yellowing.

3. Method as claimed in claim 2, further comprising the step:

g) testing the plants identified as showing the reduced susceptibility to ethylene for their resistance to Russet Spotting and/or Yellowing.

4. Method as claimed in any one of claims 1, 2 or 3, wherein the plants are leafy vegetable plants.

5. Method as claimed in claim 4, wherein the plants belong to the genus *Lactuca*.

6. Method as claimed in claim 5, wherein the plant belongs to the species *Lactuca sativa*.

7. Method as claimed in any one of claims 1-6, wherein the plant population is a population of mutant plants, a germplasm collection or a population of transgenic plants.

8. Method as claimed in claim 7, wherein the population of mutant plants is obtained by a mutagenesis treatment using chemicals and/or irradiation.

9. Method as claimed in any one of claims 1-8, wherein the concentration of ethylene in step b) is at least 10 µg/litre.

10. Method as claimed in claim 9, wherein the concentration of ethylene in step b) is between 11 and 25 µg/litre.

11. Method as claimed in any one of claims 1-10, wherein the concentration of ethylene in step e) is about 4 to 5 µg/litre.

12. Use of a lettuce plant of the species *Lactuca sativa* showing a reduced susceptibility to ethylene and a physiological disorder that is induced by ethylene as compared to a control plant, seed of which plant is deposited with the NCIMB on 3 January 2007 under accession number NCIMB 41449, NCIMB 41450, NCIMB 41448, NCIMB 41451, NCIMB 41452 or NCIMB 41453, as a crop.

13. Use of a lettuce plant as defined in claim 12 as a source of seed.

14. Use of a lettuce plant as defined in claim 12 or a part thereof as a source of propagating material.

15. Use of a lettuce plant as defined in claim 12 or a part thereof for consumption.

16. Use of a lettuce plant as defined in claim 12 for conferring the trait of the reduced susceptibility to ethylene and the physiological disorder on another lettuce plant.

17. Use of seeds of which a representative sample was deposited under accession numbers NCIMB 41449, NCIMB 41450, NCIMB 41448, NCIMB 41451, NCIMB 41452 and NCIMB 41453 for transferring the trait of reduced susceptibility to ethylene and a physiological disorder that is induced by ethylene into another agronomically valuable lettuce plant of the species *Lactuca sativa*.

18. Use as claimed in claim 17, wherein the physiological disorder is Russet Spotting and/or Yellowing.

19. Use as claimed in claim 14 or 15, wherein the part of the plant is selected from leaves, heads, cut-leaves, processed heads and baby leaves.

20. Use of seeds as defined in claim 17 to produce a vegetable product, which vegetable product is lettuce.

CA 02636110 2008-07-02

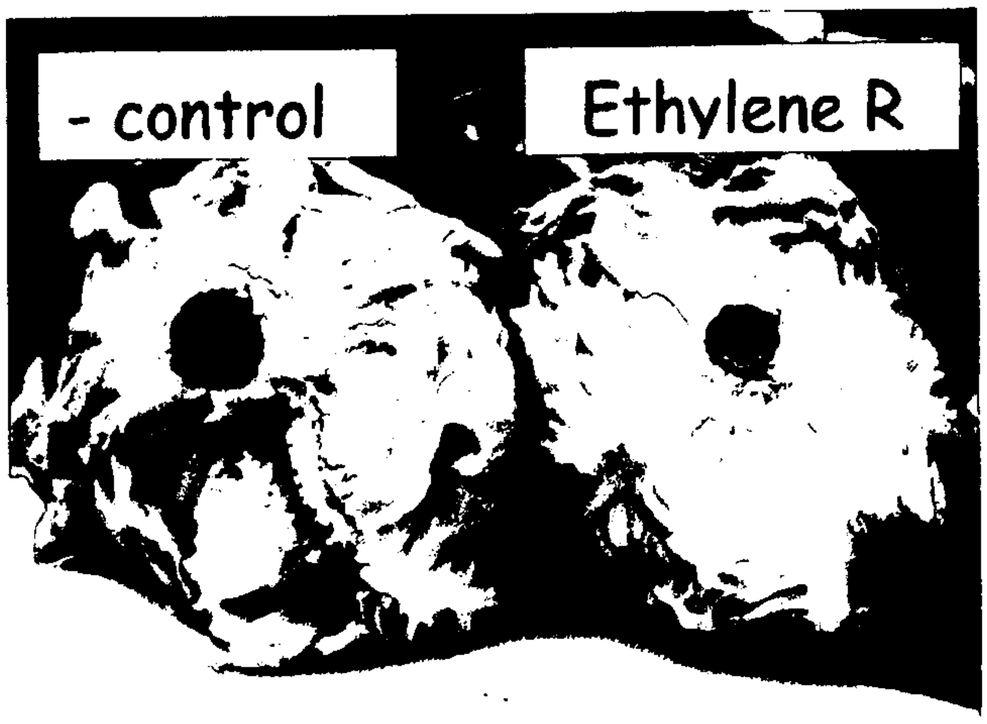

FIG. 1A

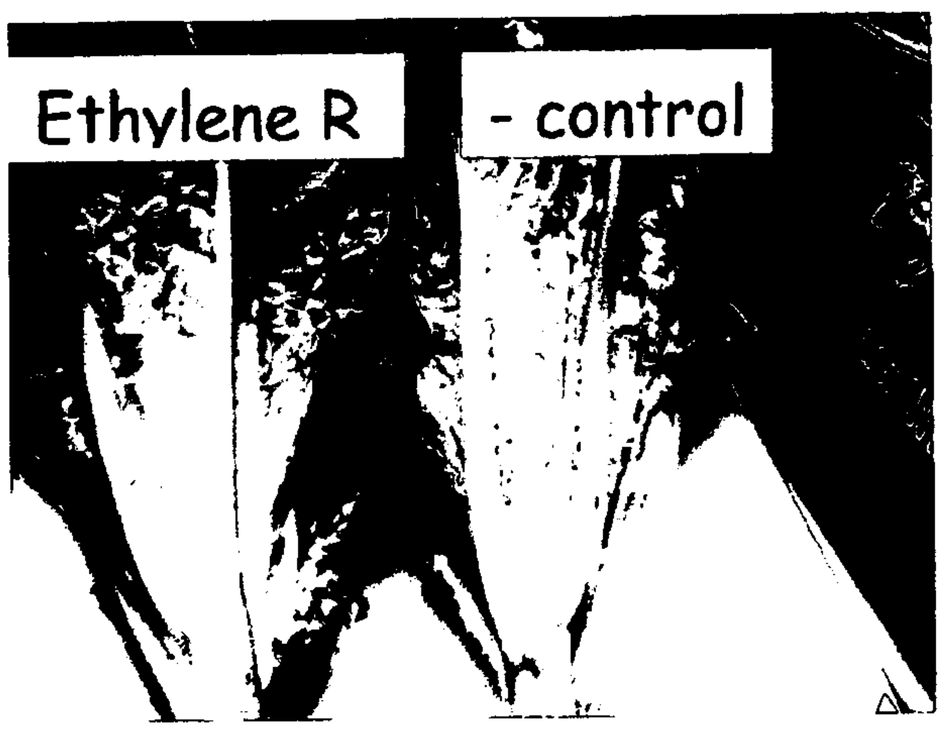

FIG. 1B