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(54) **SCREENING METHOD FOR SELECTING PLANTS THAT SHOW A REDUCED WOUND-INDUCED SURFACE DISCOLOURATION**

SCREENING-VERFAHREN ZUR AUSWAHL VON PFLANZEN, DIE EINE VERMINDERTE VERWUNDUNGSSTIMULIERTE OBERFLÄCHENVERFÄRBUNG AUFWEISEN

PROCÉDÉ DE CRIBLAGE POUR SÉLECTION DE PLANTES PRÉSENTANT UNE DÉCOLORATION SUPERFICIELLE RÉDUITE APRÈS BLESSURE

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Description

Field of the invention

[0001] The present invention relates to a method for screening a population of plants or plant parts for the presence therein of individuals that show a reduced wound-induced surface discolouration as compared to a control plant or plant part.

Background of the invention

[0002] Due to increasing demand, processing of fresh produce, such as lettuce, radicchio, endive, and other vegetables, has expanded significantly over recent years. The harvesting and processing of leafy vegetables involves extensive cutting of the leaves, which induces a strong wound response. This wound response leads to a rapid deterioration of the processed product. This deterioration is manifested by discolouration due to enzymatic browning or pinking at and around the wound surface, respiration and desiccation due to transpiration. Especially the enzymatic browning or pinking is considered of significant importance, determining directly or indirectly the overall quality of the fresh cut, packaged leafy vegetables, like lettuce and radicchio.

[0003] Moreover, as a consequence of the deterioration, micro- organisms can significantly increase in number, which may compromise food safety. The highly perishable nature of processed lettuce leads to a strong off- colour, off- odour and off- texture perception by the consumer which is hampering a faster than current growth of the so- called convenience market.

[0004] Other vegetables, like potatoes, mushrooms, celeriac, artichoke and egg plant, but also fruits and flowers may be subject to undesirable discolouration. For example, fruits like banana, apple, pear, avocado, mango, peach and apricot, etc. quickly turn brown when sliced or peeled. Measures have to be taken when offering these fruits in processed form, such as sliced, diced, peeled or in fruit salads.

[0005] Cut flower stems, for example from gerbera or chrysanthemums, may also be prone to discolouration which is undesirable from a commercial point of view because discolourations are considered to be unattractive by the consumer thus reducing the product's marketability.

[0006] In order to inhibit the deterioration process in vegetables such as lettuce, many chemical or physical post-harvest treatments have been developed which can be applied to decelerate the deterioration of the processed lettuce.

[0007] Amongst these are the packaging of fresh cut leafy vegetables under a modified atmosphere, application of edible coatings, heat shock treatment and addition of chemicals, which inhibit the enzymatic browning.

[0008] When fresh cut lettuce is packaged under an atmosphere of reduced oxygen at low temperatures, the enzymatic browning can substantially be reduced. However such modified, low oxygen environment leads to anaerobic respiration, which creates an off-flavour and off-odour of the produce which is perceived as very unattractive.

[0009] Edible coatings are thin layers of materials, which act as physical insulation barrier and which effectively protect the produce from different forms of deterioration such as evaporation and browning. These coatings can for example be made of resins, polysaccharides or protein.

[0010] It has further been demonstrated that browning of fresh cut lettuce can be prevented by applying a brief heat shock of 90 seconds at 45°C, immediately after processing. Possibly, the heat shock diverts protein biosynthesis from the enzymes involved in discolouration towards heat shock proteins thereby reducing the enzymatic browning capacity. Alternatively, the effect of heat shock treatment on browning may be explained by thermosensitivity of enzymes involved in the discolouration pathway.

[0011] Chemicals, which can be applied can for example be reducing agents like vitamin C, chelating agents like EDTA, complexing agents like cyclodextrin and enzymatic inhibitors like L- cysteine. Application of chemicals in fresh food obviously involves food safety issues and requires regulatory approval. Combinations of the post- harvest technologies described above can be thought of and ultimately the applied procedure is a trade- off between technological efficacy, cost and food safety.

[0012] Irrespective of the technology applied, improvement of post-harvest quality of processed vegetables, fruits an flowers will come at a cost and therefore a clear need in the art exists to provide alternatives, which eliminate or reduce the need to apply physical or chemical post-harvest technologies.

Summary of the invention

[0013] It is the object of the present invention to provide a screening method to select for plants, that show a reduced wound-induced discolouration response to provide plants and progeny derived therefrom that are resistant to post-harvest processing disorders such as enzymatic browning or pinking. Discolouration upon wounding can also be visible in parts of the plants, such as stems, seeds, fruits, leafs, flowers, tubers, shoots, etc. It is thus a further object of the

invention to provide a screening method to select for plants, that show a reduced wound-induced discolouration response in their plant parts.

[0014] The invention thus provides a method for screening a population of plants or plant parts for the presence therein of individuals that show a reduced discolouration as compared to a control plant, which method comprises

a) providing a population of plants or parts of the plants from the population;
b) incubating the plant or plant parts having a wound surface created thereon in an aqueous environment to allow for discolouration to occur therein or thereon;
d) observing the discolouration in or on the plants or plant parts;
e) comparing the observed discolouration with the discolouration that is observed in the control plant or plant part to identify plants or plant parts that show no discolouration or a discolouration that is reduced as compared to the control plant or plant part, wherein the plant or plant parts used in the screen are young plant material.

[0015] The method of the present invention has two main embodiments. In the first embodiment the discolouration is the result of the conversion of an endogenous substrate. Such discolouration will arise spontaneously upon incubation of the plant or plant part in a certain environment for a certain amount of time. The discolouration in this case is wound-induced. The invention particularly relates to the naturally occurring enzymatic pinking and browning reactions. The screening method of the invention is intended to identify plants that do not show this reaction or show a reduced reaction as compared to a control.

[0016] In the second embodiment the discolouration is caused by the conversion of an exogenously added substrate that can be converted into a coloured substrate that becomes visible when the reaction in the plant occurs. Such colour reaction may or may not be wound-induced. It occurs for example also in the seeds coats of intact seeds. The screening method of the invention is intended to identify plants that do not show this reaction or show a reduced reaction as compared to a control.

[0017] The latter embodiment relates more in particular to a method for screening a population of plants or plant parts for the presence therein of individuals that show a reduced discolouration as compared to a control plant or plant part, which method comprises

a) providing a population of plants or parts of the plants from the population;
b) incubating the plants or plants parts with a substrate that can be converted into a coloured pigment to allow for discolouration to occur therein or thereon;
c) observing the discolouration in or on the plants or plant parts;
d) comparing the observed discolouration with the discolouration that is observed in the control plant or plant part to identify plants or plant parts that show no discolouration or a discolouration that is reduced as compared to the control plant or plant part, wherein the plant or plant parts to be screened are young plant material, seeds or germinated seeds.

[0018] The method of the invention can be used for any plant that may be subject to discolouration, but is in particular useful for produce, in particular vegetables or fruits, or for flowers. The method is *inter alia* suitable for leafy vegetables, such as lettuce, radicchio or endive, for tubers, like potato or sweet potato, for roots, such as celeriac, for shoots, such as witloof, or for mushrooms. The method can furthermore be used for fruits, such as apple, banana, avocado, peach, pear, apricot, mango, eggplant and for flowers or flower stems, such as gerbera stems, chrysanthemum flowers, artichoke bottoms etc.

[0019] The screening method of the invention is intended for identifying plants that have a reduced wound-induced surface discolouration in one or more of their parts or tissues. For the screening, it is therefore very practical to use the part or tissue that is prone to discolouration. In lettuce, this may be the leaf or a part thereof, such as a leaf punch, in banana slices of the peeled fruit can be suitably used and in flowers slices of the stem are a very practical test vehicle.

[0020] However, it has been found that discolouration can also be tested on tissues that are not wounded. In the Examples it is shown that also intact seed coats and root tips are capable of inducing a colour reaction in the presence of an exogenously added substrate that can be converted into a coloured pigment without being wounded. The reduction or absence of this colour reaction can be used in screening for plants that have a reduced discolouration.

[0021] In a specific embodiment the method is in particular useful for selecting plants belonging to the family *Asteraceae,* in particular plants of the genus *Lactuca* and more in particular to the species *Lactuca sativa* or plants belonging to the genus *Cichorium* and in particular to the species *Cichorium intybus* and *Cichorium endivia* that show an absence or reduction of wound-induced surface discolouration.

[0022] The plant population that is screened with the method of the invention can be any plant population, but is preferably a variable plant population that has many different members to increase the chances of finding a plant that shows a reduced wound-induced discolouration. Such a variable population can be made by means of a mutagenic

treatment using for example chemicals and/or irradiation and is then called herein a mutant plant population. Alternative populations are germplasm collections, which are collections of plants showing natural variation. Also, a population of transgenic plants can be used.

[0023] The method of the invention is suitably performed with plant parts having a wound surface. Very useful test samples are discs that are punched from a leaf, the so-called leaf discs. Alternatively, the midrib tissue of veined leafy vegetables can be used. Suitably, discs are cut from such ribs. In fruits the cut surfaces of halved fruits may be assessed or alternatively slices or dices. For flowers, slices of the stem are a very useful test sample.

[0024] Incubation takes place in an aqueous environment. The method of the invention can be very well practised with leaf discs that are incubated on or between wetted filter paper. The colour response is then very well visible around the edges of the wound on the paper.

[0025] Alternatively, the aqueous environment comprises water or a solution. In a specific embodiment that will be further illustrated below the solution contains a substrate, such as L- 3, 4- dihydroxyphenylalanine. This compound is converted to the production of the black pigment melanin by the enzyme polyphenol oxidase. Alternative compounds which can be used for screening lettuce plants in this respect include but are not limited to chlorogenic acid, isochlorogenic acid, L- tyrosine and catechol.

[0026] The invention can further be performed with progeny of a parent plant that shows the absence or reduction of wound-induced leaf discolouration to demonstrate that the progeny still has the same absence or reduction of wound-induced leaf discolouration as found in the parent plant.

[0027] The invention can also be performed on parts of the plants. The plants parts like lettuce or endive heads or leaves are usually the parts that have a cut surface that may be subject to discolouration. Other parts are fruits, shoots, roots, seeds, tubers, flowers, stems, etc.

[0028] In a further embodiment of the invention seeds or germinated seeds can be used as the vehicle on which the screening method is performed in the presence of an exogenously added substrate. In the case of germinated seeds the young root tips are involved in the colour reaction.

[0029] The invention is commercially very interesting for identifying mutant plants that can be used in the processed vegetable market. As explained above, discolouration of produce, in particular fresh fruits and vegetables, is considered undesirable since the discoloured product is rejected by the consumer.

Detailed description of the invention

[0030] When lettuce is harvested and processed by cutting, many leaf wound surfaces are generated which leads to a significant response of the plant or plant parts, manifested by a brown or pink discolouration at or adjacent to the wound surface. Pinking can also be observed at sites distant from the wound surface at the midrib of the leaf as well as the butt. Sometimes pinking can also be observed at stages just prior to harvest which is considered to be due to abiotic stress or over-maturity of the crop.

[0031] Other plants, in particular other vegetables, fruits and flowers can also be prone to discolouration. The method of the invention is thus also a very convenient screen to identify other plants, in particular other vegetables, or fruits or flowers that show a reduced wound-induced discolouration response.

[0032] The different forms of discolouration are effected by enzymatic activity, which is strongly enhanced as a consequence of wounding and which generates several forms of polyphenols and reaction products derived therefrom.

[0033] An important enzymatic activity involved in the browning reaction is PPO. PPO activity in relation to enzymatic browning is not restricted to lettuce but has been described for many other plant species to be involved in post-harvest deterioration like in apple, banana and potato. In fact, PPO is widely recognised to be one of the most important enzymes involved in post-harvest deterioration of many processed fresh fruits and vegetables.

[0034] For this reason PPO has been the target of many technologies, which aim at the reduction or prevention of its activity in order to increase post-harvest quality of food products. PPO catalyses a reaction in which polyphenols residing in the plant tissue are oxidised to give rise to the formation of o-quinones. Subsequently, enzymatic and non-enzymatic reactions lead to the formation of brown or black pigments.

[0035] In many plant species PPO is encoded by a small gene family of which the individual members may have different temporal and spatial expression patterns indicative of functional divergence. It has for example been shown that lettuce contains different PPO isoforms in the photosynthetic and vascular tissue of the leaf.

[0036] The natural substrate of PPO can differ between the different species. **Table 1** lists the PPO substrates for various vegetables and fruits that are subject to discolouration upon wounding. These and other substrates can be used in a exogenous substrate-based screening method of the invention.

Table 1

Source	Phenolic substrates
Apple	chlorogenic acid (flesh), catechol, catechin (peel), caffeic acid, flavonol glycosides, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxy benzoic acid, p-cresol, 4-methyl catechol, leucocyanidin, p-coumaric acid
Apricot	isochlorogenic acid, caffeic acid, 4-methyl catechol, chlorogenic acid, catechin, epicatechin, pyrogallol, catechol, flavonols, p-coumaric acid derivatives
Avocado	4-methyl catechol, dopamine, pyrogallol, catechol, chlorogenic acid, caffeic acid, DOPA
Banana	3,4-dihydroxyphenylethylamine (Dopamine), leucodelphinidin, leucocyanidin
Eggplant	chlorogenic acid, caffeic acid, coumaric acid, cinnamic acid derivatives
Lettuce	tyrosine, caffeic acid, chlorogenic acid derivatives
Mango	dopamine-HCl, 4-methyl catechol, caffeic acid, catechol, catechin, chlorogenic acid, tyrosine, DOPA, p-cresol
Mushroom	tyrosine, catechol, DOPA, dopamine, adrenaline, noradrenaline
Peach	chlorogenic acid, pyrogallol, 4-methyl catechol, catechol, caffeic acid, gallic acid, catechin, dopamine
Pear	chlorogenic acid, catechol, catechin, caffeic acid, DOPA, 3,4-dihydroxy benzoic acid, p-cresol
Potato	chlorogenic acid, caffeic acid, catechol, DOPA, p-cresol, p-hydroxyphenyl, propionic acid, p-hydroxyphenyl pyruvic acid, m-cresol
Sweet potato	chlorogenic acid, caffeic acid, caffeylamide

[0037] In many plants, the level of PPO enzyme is not specifically induced upon wounding of plant tissues but it resides inactively in the chloroplast. Upon wounding PPO is activated which is manifested due to the fact that the phenolic substrate residing in the vacuoles is brought into contact with PPO due to tissue disruption.

[0038] In lettuce, the production of polyphenols, which are the substrate of PPO, is induced upon wounding. Therefore, the browning potential of lettuce tissue seems not to be limited by the amount of PPO in the leaf tissue but rather by the rate of polyphenol biosynthesis upon wounding.

[0039] In this respect the situation may differ between crops. For example, in apple the amount of polyphenols is sufficient to generate a browning response of the fruits within one hour after wounding whereas in lettuce the browning reaction may take a few days due to the fact that in lettuce the polyphenol pool largely needs to be synthesised de *novo* upon wounding.

[0040] The synthesis of polyphenols occurs through a well-characterised biochemical pathway called the phenylpropanoid pathway. The first, committed step of this pathway is catalysed by the enzyme phenylalanine ammonia lyase (PAL, Hahlbrock, K and Scheel, D (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 347-369). PAL converts the amino acid phenylalanine synthesised through the shikimate pathway into cinnamic acid.

[0041] In lettuce, wounding of leaves leads to a strong induction of PAL gene expression and PAL activity. The formation of polyphenols is correlated with this enzymatic activity, which suggests that PAL activity induced by wounding of lettuce is an important factor responsible for browning (Campos, R. et al. (2004) Physiologica Plantarum 121, 429-438 and references therein). However, it is currently unclear which other factors determine the final outcome of the wound-induced discolouration reaction. For example, the activity of peroxidases (POD) has been suggested to be important as well in establishing the final level of discolouration (Fukumoto, L.R. et al. (2002) J. Agric. Food Chem. 540, 4503-4511; Martin-Diana A. et al (2005) Biosci. Biotechnol. Biochem. 69, 1677-1685).

[0042] As the enzyme activity depends on the availability of internal hydrogen peroxide, the contribution of POD to discolouration may be limited.

[0043] It is further evident that wounding is somehow perceived by the plant and subsequently a signal is generated through a cascade, which is currently poorly defined for lettuce. It seems obvious that these activities will primarily be targeted towards wound healing and defence against pathogens. Therefore, it is likely that many genetic factors are involved in mounting the discolouration response of wounded lettuce tissue and each of these are potential targets for genetic modification to reduce or eliminate the wound-induced discolouration.

[0044] Most of these genetic factors are currently unknown and for those known to be involved it is unclear to what extent these factors play a specific role in the discolouration reaction or perhaps have a more general function in relation to the wound physiology of the plant.

[0045] For example, although wound-induced PAL activity is considered to be determining the browning level of lettuce, products of the phenyl propanoid pathway are known to be involved in *inter alia* cell wall biosynthesis or defence response as well. Therefore reducing the wound induced PAL activity in order to reduce browning potential may compromise other functions besides wound-induced browning which may be less desirable in relation to other aspects of lettuce cultivation.

[0046] Likewise, PPO activity has been implied to be involved in defence response and therefore reducing the browning potential by reducing PPO levels may increase the susceptibility to pathogens (Thipyapong, P. et al (2004) Planta 220, 105- 117) . Therefore, it was reasoned by the inventors that a more unbiased approach may be more successful in this respect. Such approach comprises the following steps:

1. Generation of a variant population of plants, in particular a mutant population. Such mutant population can be generated by treatment of seeds or plant tissues with mutagenic agents like ethyl methane sulfonate (ems) or x-rays.
2. The set-up of an efficient phenotypic screen in which selection is based on a discolouration of the plant, in particular a wound response-induced discolouration of the plant, which is channelled through PAL and/or PPO.
3. Characterisation of the mutants modified in their wound-response with respect to post-harvest discolouration potential and absence of pleiotropic effects of the modification, which compromise growing and processing of the plant according to common practice.

[0047] The invention thus relates to a screening method for identifying, selecting and obtaining a plant showing a reduced wound-induced discolouration and post-harvest processing disorders such as enzymatic browning or pinking. In the screening method, discolouration may be observed at a wound surface but it was also found that intact tissues also show a colour reaction upon addition of a substrate.

[0048] A mutant plant population for use in the method of the invention can for example be prepared as follows:

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) optionally repeating step b) and c) n times to obtain M1+n seeds;
d) germinating the thus obtained M1+n seeds and growing the plants from those seeds.

[0049] According to the invention these plants are subsequently assayed for their wound-induced discolouration response. Plants that do not show or show a reduced discolouration response to wounding are selected. Then, progeny of the selected plants is grown and the wound-induced discolouration response is measured.

[0050] In order to create genetic variability use can be made of mutagenesis. Several chemical or physical treatments are know to the person skilled in the art which can be used to induce genetic mutations in plant species. For example, one can treat seeds in a solution containing different concentrations of a mutagen like ems. Ems alkylates primarily G residues of a DNA strand, which during DNA replication causes pairing with T instead 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.

[0051] 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. Seeds, which have been treated with a mutagenic agent 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 haploinsufficient thereby causing sterility or which induce embryo lethality will not be transferred to the M2 generation.

[0052] A similar procedure as described above for the use of ems applies for other mutagenic agents as well. Suitable mutagenic agents are well- known in the art. Particularly useful are alkylating mutagenic agents, such as diethyl sulfate (des), ethyleneimine (ei), propane sultone, N- methyl- N- nitrosourethane (mnu), N- nitroso- N- methylurea (NMU), N- ethyl- N- nitrosourea (enu), sodium azide.

[0053] Alternatively, the mutations are induced by means of irradiation, which is for example selected from x- rays, fast neutrons, UV irradiation.

[0054] 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, gene targeting, introduction of modified target genes which compete with the endogenous product, downregulation through RNA interference, etc.

[0055] The M2 population of a mutagenesis treatment can be used in screening procedures aimed at wound response, which is channelled through PAL and PPO. It is obvious to the skilful artisan that any population of plants, which carries genetic variation can be taken as starting material for such phenotypic screen, such as germplasm collections, which are collections of plants showing natural variation or populations of transgenic plants.

[0056] Production of M1 and M1+n seeds is suitably effected by means of self-pollination.

[0057] In order to carry out the phenotypic screen of the invention, a wound surface must be generated as the enzymatic discolouration reaction is induced upon wounding. Such wounding may be achieved by cutting, punching, slicing, abrasing, squashing, breaking, peeling, crushing, pressing, slashing, grinding, fluid injection, osmotic shock, detaching, mowing, tearing.

[0058] It was found that the method of the invention in which an exogenously added substrate is used, can also be

practised on intact tissues and plant parts, such as seeds, in situations where PPO is within reach of the substrate, for example when PPO is secreted or located outside the cell. The enzyme that induces the colour reaction is then also available without prior wounding.

[0059] After wounding or when no wounding is needed, a phenotypic characteristic must become manifest which is diagnostic for the pathway leading to tissue discolouration and which can be used very efficiently in a screen of the mutant population.

[0060] It was surprisingly found that such phenotypic characteristics can be obtained by taking plant parts and incubating them under very specific conditions which favour different forms of discolouration, in particular wound surface discolouration, to occur. Subsequently, such assays can be applied to large numbers of plants or plant parts, such as mutant plants, in order to select those plants which show a reduction of the wound-induced discolouration response.

[0061] One embodiment of this invention is based on the surprising finding that when discs from leaves, such as lettuce leaves or leaves of endive or witloof, are taken and incubated between wetted filter papers at 5°C, after approximately 4 days the formation of a pink dye at the edges of the leaf discs becomes apparent. Suitable filter paper is filter paper type 1450 CV, Ref.no. 10 313 281 from Schleier & Schuell, Microscience GmbH, Dassel, Germany. Upon further incubation, the signal intensifies and after approximately one week the maximum intensity has been reached. The formation of the pink dye occurs specifically at wound surfaces.

[0062] The discolouration can be measured by scoring on a visual scale from 0, which means no browning or pinking, to 10, which means browning and pinking like a standard variety of the plant to be screened (for example *L. sativa* for screening lettuce). In the Examples regarding lettuce the *L. sativa* variety 'Troubadour' is used as a standard for 10.

[0063] If desired, pictures can be used for comparison to score the intermediate classes between 0 and 10. In addition, digital pictures can be made of the filter paper with the pink or brown dye, followed by counting per leaf disc position the number of pixels with an intense pink or brown colour. 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 pinking or browning than the standard. The applied significance level of a one-sided test is 0.001.

[0064] For mutants, the statistical comparison can be made between the pinking scores of the original variety, which is the best available standard, and the pinking scores of the individual mutants and/or their offspring.

[0065] For finding the trait of the invention in existing plants, representative samples of varieties, breeding lines and/or gene bank accessions can be used. The statistical comparison can then be made between the pinking scores of the individual accession under investigation and the rest of the population. When statistically testing individuals for significantly less pinking 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)).

[0066] Further, it was shown that this response can be obtained using many different types of tissue of leaves of different developmental stages. For example, in lettuce midrib tissue can also be induced to give this response upon wounding. When applied to different types of lettuces, such as butterhead, iceberg, cos, batavia or oakleaf, no individual accessions were found that showed significantly less pinking than the rest of the investigated population. It is therefore concluded that within the cultivated lettuce types there is no or only very limited genetic variation for wound- induced pink discolouration.

[0067] It was further demonstrated according to the invention that a specific inhibitor of PPO, L-cysteine, when applied during the reaction, strongly suppressed the formation of the pink dye. In addition, it was found that the formation of the pink dye was inhibited by cinnamaldehyde, which is an inhibitor of PAL activity and browning of fresh cut lettuce (Fujita, N. et al (2006) Biosci. Biotechnol. Biochem. 70, 672-676). These findings show that the pink discolouration response of lettuce is PAL and PPO dependent.

[0068] Enzymatic browning of fresh cut lettuce is known to be very effectively prevented by the application of a brief heat shock. The observed effect can be explained by assuming re-routing of protein biosynthesis from the phenyl propanoid pathway towards heat shock proteins thereby reducing the metabolic flux towards the formation of polyphenols.

[0069] Alternatively, the effect may be explained by assuming that the enzymes involved in polyphenol oxidation, such as PPO and POD, are inactivated by the heat shock treatment. When the heat shock is applied to lettuce, which is subsequently assayed for the pinking response, it was shown that this response, like the enzymatic browning, was effectively inhibited. This demonstrates that the pinking response of lettuce, which is part of this invention is physiologically very similar to the well known enzymatic browning response.

[0070] This finding was further substantiated by applying L-cysteine as a reducing agent. L-cysteine, besides being an inhibitor of PPO, is also known to react with coloured o-quinones and convert them back into colourless diphenols in a chemical reduction reaction. When the pink dye formed by lettuce leaf discs is treated with L-cysteine, it was demonstrated that the pink compound was converted into a colourless compound. It seems therefore likely that the pink dye is an o-quinone formed by PPO.

[0071] This was corroborated by the finding that reducing agents like ascorbic acid or glutathion also convert the pink dye into a colourless compound.

[0072] In addition, when plants, which are taken from the field, which show pinking, are treated with L-cysteine, the

pink discolouration is also eliminated. This demonstrates that the leaf disc pinking response is representing the naturally occurring pinking phenomenon, which can sometimes be seen on plants growing under field conditions.

[0073] A further embodiment of this invention is based on the following experiment. Parts of a lettuce leaf of a head are produced by cutting and incubated at 16°C in air. As a response the wound surface turns brown after approximately 4 days. Especially at the wound surface of the main vein the browning can clearly be observed. Furthermore, the browning reaction can also be observed at the whole plant level upon damaging leaves by cutting or abrasion.

[0074] All of these browning reactions can completely be inhibited by L-cysteine, an inhibitor of PPO, which demonstrates that these phenotypes are manifested through PPO activity and therefore can be considered diagnostic for post-harvest browning as observed during processing and packaging of lettuce.

[0075] These wound-induced browning reactions can be generated in an efficient manner which can be exploited in a phenotypic screening procedure to identify mutant plants which are reduced in wound-induced browning potential.

[0076] A further embodiment of this invention is based on the discolouration at wound surfaces of lettuce tissues or the colour reaction observed in, on or near intact lettuce tissues, such as lettuce seed coats, induced by applying substrates which can be converted by the phenol oxidising enzymes into coloured compounds.

[0077] For example, when lettuce leaf discs are incubated with the PPO substrate L- 3, 4- dihydroxyphenylalanine (L-DOPA), a dark brown to black discolouration is observed at the wound surface which is the manifestation of the formation of melanine through PPO. When L- cysteine was applied simultaneously, the black discolouration was completely inhibited which confirms the assumption that this discolouration is PPO mediated.

[0078] Although L-DOPA is not considered to be a natural substrate for lettuce PPO it can be useful in assays aimed at the identification of mutants, which show reduced wound-induced discolouration.

[0079] In a similar manner as described for L-DOPA other substrates can be applied in order to raise a discolouration response. These include but are not limited to chlorogenic acid, isochlorogenic acid, L-tyrosine, and catechol.

[0080] Taken together, the formation of the different dyes at wound surfaces generated in plants monitors modifications in a pathway starting by the induction of a wound signal, channelled through PAL and PPO and leading to discolouration. As described, these wound-induced discolouration reactions can readily be assessed by visual inspection which allows a very efficient mutant screening procedure. The rationale underlying the method described by this invention is illustrated in **Figure 1.**

[0081] According to this invention it was thus found that the wound-induced discolouration pathway of leaf discs *in vitro* largely overlaps with the wound-induced discolouration of lettuce processed at industrial scale and can therefore be considered diagnostic for this process. This is corroborated by the notion that inhibitors of PAL or PPO inhibit the enzymatic browning of processed and packaged lettuce under practical, industrial conditions. Importantly, as the procedure comprises the inducing step i.e. wounding and one of the final metabolic conversions mediated by PPO, the procedure allows to capture all genetic factors directly or indirectly involved in this physiological process. Moreover, as this response can be generated using a whole range of leaf tissues of leaves of different developmental stages, mutant screens can be targeted towards these different stages or tissues when considered relevant.

[0082] Mutant plants, which have been identified as being modified with respect to the physiological process leading from wounding to a PAL- and PPO-dependent discolouration based on one or more of the phenotypic assays described above can be further characterised. Such characterisation can be done at different levels e.g. at the molecular, biochemical, physiological and phenotypic level.

[0083] It is obvious to those skilled in the art that variable levels of discolouration may be observed which may reflect either the presence of different mutant loci or different allelic forms of identical loci affecting the discolouration trait in the original population.

[0084] In case of recessive mutations these two possibilities can easily be distinguished by carrying out allelism tests, which comprise the crossing of the two mutant plants and determining the phenotype of the hybrid. In case of allelism of the mutations, the reduced discolouration trait 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.

[0085] As in one embodiment of the invention random mutagenesis is applied to generate the starting population, mutations in the genetic background may also contribute to the variation of the phenotype under the experimental conditions. In order to discriminate between single mutations of different strengths and a combined effect of mutations in the genetic background, backcrosses should be performed to create uniform genetic backgrounds for the different reduced discolouration events.

[0086] Such procedure is further relevant in order to determine whether mutations at specific loci involved in wound-induced discolouration display pleiotropic effects.

[0087] The M2 plants thus selected on the basis of a reduced discolouration response are used to grow M3 seeds. Subsequently, the inbred lines descending from the reduced discolouration events are re-evaluated for their reduced response wounding. In addition, the reduced browning or pinking can be assessed in different genetic backgrounds and under different conditions of crop cultivation and processing. The screening methods of the invention can be used for all these assessments.

[0088] Biochemical studies can be performed to address questions related to the pathways affected by the genetic modification. Molecular studies can be performed to determine if candidate genes putatively involved in the enzymatic browning or pinking response like genes encoding PAL, PPO or peroxidases have been modified. Genetic analysis can further be carried out to demonstrate if the modification found in a candidate gene is causative with respect to the altered phenotype.

[0089] Although induced mutagenesis is the preferred method of providing a plant population to be used in the screening method of this invention, it is known to the person skilled in the art that technology exists which allows to modify gene targets residing in the genome of a plant in a specific manner. For example, chimeric oligonucleotides have been demonstrated to be effective mutagens with a specific mode of action.

[0090] 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.

[0091] In case mutagenic oligonucleotides, gene targeting or transgenic approaches are used to modify a genetic factor involved in wound-induced discolouration response, obviously, the primary structure of the relevant genes should be known.

[0092] When progeny plants of particular mutants grown from seeds obtained through self-fertilisation are assayed for pinking, a similar reduction as found for the originally identified mutant is observed. This demonstrates that a reduced pink discolouration response can be heritable and caused by a modification of the genome.

[0093] A further surprising finding was the fact that when progeny plants of mutants identified as showing a reduced wound-induced discolouration are grown to maturity and tested for enzymatic browning of wounded midrib tissue, this response is also strongly inhibited. This shows that the leaf disc pinking assay is causally related to enzymatic browning in lettuce and that the pinking assay can be used to predict the level of enzymatic browning of a mature lettuce plant.

[0094] Therefore, the leaf disc pinking assay can be used as a selection tool to identify lettuce plants with a reduced enzymatic browning potential. Such tool can be used to identify lettuce plants with reduced enzymatic browning potential from any kind of plant population irrespective of the cause of the genetic variation, which resides in such population. For example, in addition to ems populations, one can use natural accessions or breeding populations.

[0095] The screening method can also be used for screening other plants that show wound-induced discolouration.

[0096] One or more of the screening methods provided by this invention can for example be applied to any plant species for which post-harvest processing quality needs improvement. In addition to cultivated lettuce this invention can also be applied to other plant species, for example belonging to the *Asteraceae,* such as wild species of the *Lactuca* genus or plant species belonging to the plant genus *Cichorium* to which species like endive (*Cichorium endivia),* chicory and witloof chicory (*Cichorium intybus*) belong. Furthermore, other crop plants such as apple, radicchio, potato, sweet potato, celeriac, mushrooms, banana, avocado, peach, pear, apricot, mango, eggplant, and for flowers or flower stems, such as gerbera stems, chrysanthemum flowers, artichoke bottoms etc. can be screened with the methods of the invention.

[0097] The present invention relates to a method for detecting a phenotypic feature in a plant by performing one of the screening methods that are disclosed herein. The presence of the feature is determined by means of one or more of three discolouration tests, namely the occurrence of pinking or browning or the ability to convert a substrate into a coloured pigment, such L-DOPA into melanin.

[0098] The "control" as used herein is any plant of which it is known that it shows one or more of the discolouration reactions pinking, browning and conversion of L-DOPA to melanin, which reactions can be inhibited by L-cystein or cinnamaldehyde. Suitably a plant is used of which a leaf disc or other plant part when incubated between wetted filter paper at 5°C for 7 days shows pink discolouration around the edges of the disc or part.

[0099] The present invention will be further illustrated in the Examples that follow and that are not intended to limit the invention in any way. The Examples refer to lettuce leaf discs and seeds, *Cichorium* and eggplant, but instead of lettuce other plants or parts thereof, in particular fresh fruits and vegetables, can be used. In the Examples reference is made to the following figures.

Figure 1: Schematic outline of the rationale behind the design of the mutant screening procedure of lettuce populations for reduced post-harvest enzymatic discolouration. The input signal of the screen is wounding of leaf tissue, which is sensed by the plant and which generates a divergent signalling response leading to a number of physiological processes including senescence, respiration and tissue discolouration. This input signal can be combined with the application of phenolic compounds as PPO substrates.
The output signal of the screen is a brown or pink discolouration, depending on the conditions applied, of the wound surface diagnostic for post-harvest browning and pinking. This is inferred from the fact that the output signal is completely inhibited by cinnamaldehyde and L-cysteine, which are specific inhibitors of PAL and PPO, respectively.

Figure 2: Representative image of the output phenotype of the screen based on pink discolouration of leaf discs. Leaf discs of lettuce plants (1 disc per plant) are arrayed between wetted filter papers and incubated at 5°C for 7 days. A pink discolouration can clearly be observed around each leaf disc at the wound surface.

Figure 3: The leaf disc pinking assay (4 discs per dish) carried out in the presence of different concentrations of the PAL inhibitor cinnamaldehyde. The number above each dish shows the % of cinnamaldehyde used.

Figure 4: Inhibitory effect of the PPO inhibitor L-cysteine on the pink discolouration of lettuce leaf discs (4 discs per dish)incubated between wetted filter papers. The number above each dish shows the % of L-cysteine used.

Figure 5: Inhibitory effect of the PPO inhibitor L-cysteine on the black discolouration of lettuce leaf discs (4 discs per dish)incubated between wetted filter papers in the presence of 1.5 mM L-DOPA. The number above each dish shows the mM concentration of L-cysteine used.

Figure 6: The effect of heat shock pre-treatment on lettuce leaf disc pinking. The heat shock was applied for 90 seconds on intact leaves at the temperature indicated on each dish.

Figure 7: Conversion of the pink dye formed by the wound response of lettuce to a colourless compound by L-cysteine. The upper row of dishes shows the L-DOPA assay whereas the lower row of dishes shows the pinking assay. The lower of the two discs in each dish was treated with L-cysteine after the wound response had been completed. The concentration of L-cysteine used are 0, 0.001, 0.01, 0.1, 1 and 10 mM, which is indicated above.

Figure 8: Reduction of pinking in filed grown lettuce by L-cysteine. The upper panel shows typical pinking symptoms of a lettuce leaf taken from a plant, which was extremely stressed by water logging. The main veins show the presence of a pink dye. The lower right panel shows a disc taken from the leaf showing pinking symptoms after treatment with 1 mM L-cysteine for 30 minutes at room temperature. The lower left panel shows a similar leaf disc after treatment with water for 30 minutes at room temperature.

Figure 9: Panel A: Phenotypic analysis of individual lettuce M2 plants (grouped in pools)for leaf disc discolouration according to the method described by this invention. A total of 138 samples out of 12000 is shown in this panel of which the one indicated by an arrow showed a strongly reduced pinking discolouration. Panel B: Re-testing of the selected individual indicated in panel A confirmed the near absence of the formation of the pink discolouration (sample in the middle position) as compared to control samples which show a clear discolouration response.

Figure 10: Phenotypes of M2 lettuce plants. The plants labelled 1, 2, 4, 5, 7, 10 and 12 show reduced pink leaf disc discolouration using the assay according to this invention. Plants 3, 6, 8, 9, and 11 are plants, which showed a level of pink leaf disc discolouration comparable to the wild type control. Plant 1 is the only example of a mutant which shows a strong reduction in pink discolouration and a normal growth habitus. Plants 2, 4, 5, 7, 10 and 12 show reduced pink discolouration and a dwarfed, bleached phenotype.

Figure 11: Progeny testing of a mutant of lettuce showing a reduced discolouration. On the left, 25 control samples are shown which show a normal wound-induced discolouration response. On the right, a group of samples is shown which is taken from a series of 35 progeny plants derived from a single mutant, which is severely reduced in its wound-induced discolouration response.

Figure 12: Representative image of the output phenotype of the screen based on brown discolouration of leaf midrib pacts taken from mature Lettuce plants. The picture shows lettuce outer leaf midrib tissue discs after incubation for 3 days at 16°C. The typical brown discolouration can clearly be observed at the wound surface. Each dish contains 3 discs taken at different positions of the midrib (green, light green and white). The number above the dish indicates the mM concentration of L-cysteine which was added to the filter.

Figure 13: Conversion of L-DOPA at the lettuce leaf surface into melanin. Panel A shows the assay in a 1.5 mM L-DOPA solution. The upper tube is the negative control, the other 3 tubes are identical. Panel B shows the result of the incubation of leaf discs between wetted filter papers which contain 1.5 mM L-DOPA.

Figure 14: Progeny testing of a mutant of lettuce showing a reduced wound-induced pink discolouration on midrib browning. Panel A shows the midrib discs of 8 progeny plants, numbered 1 to 8 (3 discs per plant) of the reduced pinking mutant. Panel B shows the midrib discs of 8 control plants numbered 9 to 16 (3 discs per plant) which show a normal browning response.

Figure 15: Assessment of a mutant of lettuce showing a reduced wound-induced pink discolouration or browning response after cutting and packaging under ambient atmosphere. Leaf pieces of the head of a control plant are shown on the left and leaf pieces of the reduced discolouration mutant is shown on the right. The fresh cut leaf material was stored for 6 days at 4°C. The brown discolouration can be clearly observed in the control samples whereas the mutant samples remain unchanged.

Figure 16: Pinking assay in *Cichorium endivia* (left panel) and *Cichorium intybus* (right panel).

Figure. 17: Chlorogenic acid assay on lettuce leaf discs.

Figure 18: Browning response in cut eggplant.

Figure 19: L-DOPA assay on seeds of lettuce (top row) and egg plant (bottom row).

Figure 20: L-DOPA assay on germinated seeds of lettuce.

Figure 21: Catechol assay on germinated seeds of lettuce.

EXAMPLES

EXAMPLE 1

Genetic modification of lettuce using ems

[0100] Approximately 2000 seeds of the lettuce varieties Troubadour, Apache, Yorvik and Roderick were incubated in an aerated solution of either 0.05% (w/v) or 0.07% (w/v) ems during 24 hours at room temperature. After the ems treatment the M1 seeds were rinsed water and planted in a greenhouse at 20°C at 16 hours light, 8 hours dark regime to grow the mature plants and to induced bolting and flowering in order to produce M2 seeds. After maturation, M2 seeds were harvested, bulked and stored until further use. The mutation frequency was estimated on the basis of the relative number of individual plants with a bleached phenotype which are disturbed in the chlorophyll biosynthesis.

EXAMPLE 2

Development of a phenotypic screen diagnostic for the wound-induced discolouration of lettuce based on pink pigment formation

[0101] A phenotypic assay was developed in which leaf discolouration of lettuce induced by wounding can readily be assessed. This approach allows young plant screening for discolouration. Leaf discs of 5 mm diameter were taken from young or mature plants and placed between wetted filter papers in a tray. The system was incubated at 5°C for 7 days. During the incubation a pink dye developed at the wound site of the leaf disc which became clearly visible as a printed circle on the filter paper (**Figure 2**).

[0102] In order to demonstrate that the production of the pink dye requires an active phenylpropanoid pathway, the effect of inhibitors of PAL (cinnamaldehyde, **Figure 3**) and PPO (L- cysteine, **Figure 4**) were tested in this assay. When cinnamaldehyde was applied during the assay, the pink discolouration was completely inhibited at a concentration of 0.01% or higher.

[0103] A similar result was obtained using L-cysteine at a concentration of 0.001% and higher, while other amino acids like L-leucine or L-alanine did not show any effect. This demonstrates that L-cysteine can inhibit the pinking response of the lettuce leaf discs and that the inhibitory effect of L-cysteine is specific.

[0104] In order to demonstrate that L- cysteine is indeed acting as an inhibitor of PPO activity in this system, the lettuce leaf discs were incubated with the PPO substrate L- 3, 4- dihydroxyphenylalanine (L- DOPA) . Although L- DOPA is not considered to be a natural substrate for lettuce PPO, a dark brown to black discolouration was observed at the wound surface which is the manifestation of the formation of melanin through PPO. When 1 mM or a higher concentration of L- cysteine was applied simultaneously, the discolouration was completely inhibited as shown in **Figure 5.**

[0105] The lettuce leaf disc pinking response was further characterised by applying a heat shock before inducing the wound response. Detached leaves were incubated during 90 seconds at 21, 40, 50 and 60°C. After this treatment leaf discs were taken and assayed for pinking. The pinking response was completely inhibited when the heat shock was carried out at a temperature of 50°C or higher. This result is shown in **Figure 6**.

[0106] As L-cysteine is known to react with o-quinones, which are PPO products, by converting them back into colourless diphenols, the effects of L-cysteine on the pink dye coming from lettuce leaf discs was determined. In parallel the effect of L-cysteine was determined on the melanin formation upon incubation with L-DOPA.

[0107] Leaf discs were taken and incubated according to the procedures described above. After the wound response was completed, a concentration series of L-cysteine was added to the leaf disc and the change in colour was monitored. The result is shown in **Figure 7**. The experiment clearly demonstrated that L-cysteine was converting the pink dye back into a colourless compound whereas the black melanin formed in the L-DOPA assay was not affected by the L-cysteine. This demonstrates that the pink dye is very likely an o-quinone which is formed by the lettuce polyphenol oxidation system.

[0108] To demonstrate that the observed *in vitro* response reflects a response which is physiologically relevant the L-cysteine based discolouration was applied to field-grown plant material. This was carried out by harvesting a leaf from a field-grown lettuce plant which showed severe pinking symptoms along the veins. This is typically observed when plants have been stressed, for example by conditions of severe water logging. The leaf was used to prepare leaf discs which were incubated immediately by 1 mM L-cysteine. After approximately 30 minutes incubation at room temperature, the pink discolouration disappeared as shown in **Figure 8**.

[0109] Taken together, these experimental data show that lettuce leaf discs can be induced by wounding to produce pink discolouration which is PAL and PPO dependent. This phenotype allows an efficient and effective screening procedure for lettuce mutants which have a modified wound response induced discolouration channelled through PAL, PPO or both.

EXAMPLE 3

Screening for mutants with a reduced wound-induced discolouration

[0110] In order to identify lettuce mutants with low wound-induced enzymatic browning or pinking potential, the leaf disc assay described in **Example 2** was applied to plants of a lettuce mutant population.

[0111] 12000 Plants were grown in a greenhouse (Location: De Lier, sowing on 28 March, planting on 18 April; growing under regular lettuce grower's conditions) and from each individual plant a leaf disc was taken (sampling from 15 May onwards) and incubated as pools of (on average) 25 samples between wetted filter papers at 5°C for 7 days. A visual score was given to each leaf disc depending on the intensity of the pink discolouration. On the basis of this assessment, plants were selected of which the leaf discs showed no or a relatively low degree of wound surface discolouration. The plant with hardly visible traces of discolouration was numbered 06D.210202.

[0112] Of the 12000 plants, 1 plant was finally selected which showed only traces of discolouration which were hardly visible and 11 which showed a relatively low level of discolouration. The result of one of these assays is shown in **Figure 9.**

[0113] The discolouration assay was repeated for the initially selected 12 individuals and for most individual cases the original result was confirmed. Only the confirmed individuals were selected for further analysis and seed production.

EXAMPLE 4

Screening for mutants with a reduced wound-induced discolouration

[0114] In order to identify lettuce mutants with low wound induced enzymatic browning potential the leaf disc assay described in Example 2 was applied to plants of a lettuce mutant population. 8500 plants were grown in a greenhouse until 3 weeks old (6-8 leaf stage) and from each individual plant a leaf disc was taken and incubated between wetted filter papers at 5°C for 7 days.

[0115] A visual score was given to each leaf disc depending on the intensity of the pink discolouration. On the basis of this assessment, plants were selected of which the leaf discs showed no or a relatively low degree of wound surface discolouration. Of the 8500 plants 8 plants were selected which did not show any visible discolouration and 10 showed a relatively low discolouration. The discolouration assay was repeated for the 18 individuals that were initially selected and for most individual cases the original result was confirmed. Twelve individuals are shown in **Figure 10**. Only the confirmed individuals were selected for further analysis and seed production. One mutant plant without pleiotropic side-effectts (e.g. bleaching, dwarfing) was given number 05D.202539. The seed produced by selfing of this plant was numbered 05D.810596. The seed produced by selfing of three plants grown from seeds of 05D.810596 was numbered 07G.9979 and deposited at NCIMB. The NCIMB-number is 41441 (deposited on 10 October 2006).

EXAMPLE 5

Phenotypic analysis of the selected mutants showing reduced, wound-induced discolouration

[0116] Of the 12 mutants selected from the screen presented in **Example 3**, 6 showed a strong reduced growth phenotype and bleaching. Other mutants developed normally i.e. according to the type of the starting population of the mutagenesis experiment.

[0117] The dwarfed and bleached mutants are probably disturbed in chloroplast function. As PPO resides in these cellular organelles this may explain the relatively low response in the leaf disc assays. As such pleiotropic mutations are undesirable, these mutants were considered to be less relevant.

[0118] The mutant plant 06D.210202 which showed the strongest reduction of leaf disc discolouration showed a normal phenotype and the mutation is therefore considered specific for the discolouration without strong pleiotropic effects.

EXAMPLE 6

Confirmation of the near absence of discolouration phenotype in offspring

[0119] To demonstrate that the reduced discolouration of lettuce mutants like plant 06D.210202 from Examples 3 and 5 is caused by a genetic effect generated by the mutagenesis treatment described herein, seed were produced by selfing. The seed produced by selfing of plant 06D.210202 was numbered 06D.819784. The seeds were germinated in soil and the plants were tested for discolouration using the leaf disc pinking assay.

[0120] This experiment clearly showed that the altered phenotype had a genetic basis as all progeny plants showed a similar phenotype i.e. a strong reduction in pink discolouration, like the mutant which was used to produce the seeds.

This result is illustrated in **Figure 11**.

[0121] The seed produced by selfing of three plants grown from seeds of 06D.819784 was numbered 06D.863B2 and deposited at NCIMB. The NCIMB-number is 41454 (deposited on 3 January 2007).

EXAMPLE 7

Development of a phenotypic screen diagnostic for the wound-induced discolouration of lettuce based on brown pigment formation

[0122] Lettuce plants were grown to maturity and parts of outer leaves were taken by cutting discs from the rib tissue. The discs were incubated on wetted filter paper at 16°C. After approximately 72 hours, the wound surface had turned brown. In the presence of 10 mM L-cysteine the browning response was inhibited indicating that the observed discolouration is PPO mediated. A representative outcome of such experiment is shown in **Figure 12**.

[0123] As the response as displayed in **Figure 12** is a PPO mediated browning response, the screening procedure as described in this example can considered to be effective and unbiased in order to screen for mutants showing reduced brown discolouration which occurs during the processing of lettuce.

EXAMPLE 8

Development of a phenotypic screen diagnostic for the wound-induced discolouration of lettuce based on the conversion of L-3,4-dihydroxyphenylalanine (L-DOPA) into a black pigment called melanin

[0124] In addition to assays which address wound-induced discolouration in a broad sense, the method according to this invention also allows to screen in a more specific manner for mutants which have a reduced PPO activity. A phenotypic assay which is indicative for PPO activity was developed by using leaf discs which were incubated in the presence of 1.5 mM L-DOPA. As a black discolouration became apparent it can be concluded that L-DOPA can readily be converted by the polyphenol oxidising system at the wound surface of lettuce leaves into a black pigment called melanine. L-DOPA is converted by PPO into the reactive L-DOPA-quinone which is converted non-enzymatically via dopachrome and indol quinone into the black melanin.

[0125] Furthermore, it was shown that the reaction can be inhibited by adding 1 mM L- cysteine during the reaction (**Figure 5**) . Therefore, this assay enable the identification of mutants which are modified in the ability to mount a PPO activity at a wound surface of a leaf. The response of lettuce leaf discs to the presence of L- DOPA can be observed both in solution as well as between wetted filter papers as shown in **Figure 13**.

EXAMPLE 9

Assessing the offspring of a mutant showing a near absence of discolouration for reduced browning response *using the ribdisc browning assay*

[0126] To demonstrate that the reduced discolouration of lettuce mutants like plant number 06D.210202 from **Examples 3**, **5** and **6** which is significantly reduced in pink discolouration of wound surfaces of leaf discs is also effectively reduced in the wound-induced browning response, a number progeny plants were grown to maturity.

[0127] At this stage of development, 3 midrib discs are taken from the outer leaves of a number of progeny plants. These rib discs are incubated according to the procedure described in **Example 7.** It is shown that the progeny plants of the mutant which were earlier shown to be strongly reduced in wound-induced pink discolouration are also strongly reduced in wound-induced midrib browning. The result of this experiment is shown in **Figure 14**.

EXAMPLE 10

Assessing the offspring of a mutant showing near absence of discolouration for reduced browning response using fresh cut lettuce heads packaged in plastic bags

[0128] Mature heads of the lettuce plants grown from seed number 06D.819784 from **Example 6**, which is significantly reduced in pink discolouration of wound surfaces of leaf discs, were cut into pieces using a knife and packaged in a plastic bag containing an ambient atmosphere. Control plants which show a normal leaf disc pink discolouration response were treated in an identical manner. The bags were stored at 4°C during 6 days after which the leaf material was assessed for its browning response.

[0129] It is shown by this experiment that the progeny plants of the mutant which were earlier shown to be strongly

reduced in wound-induced pink discolouration are also strongly reduced in wound-induced midrib browning when processed and stored in plastic bags using an ambient atmosphere. The result of this experiment is shown in **Figure 15**.

EXAMPLE 11

Plinking in Cichorium

[0130] In order to identify mutants with low wound-induced enzymatic pinking potential, the leaf disc assay described in **Example 2** was applied to plants of a mutant *Cichorium* population. The results are shown in **Figure 16.** It follows that also in *Cichorium* the pinking reaction is present. The screening method of the invention is thus a powerful tool to select *Cichorium* plants that show a reduced wound-induced discolouration. Up till now the assessment of the browning response in endive and witloof could only be assessed by observing mature plants. The method of the invention which can be practised on young plant material is very efficient and fast.

EXAMPLE 12

Discolouration in eggplant

[0131] Eggplants were cut in half and incubated overnight at room temperature. From **Figure 18** it follows that the discolouration response is mainly caused by the seeds. Thus, seeds are a suitable candidate for a substrate-based discolouration assay, for example with L-DOPA.

EXAMPLE 13

Phenotypic screen diagnostic for the wound-induced discolouration of lettuce based on the conversion of chlorogenic acid

[0132] Leaf discs of lettuce were incubated with 10 mM chlorogenic acid on filter paper. **Figure 17** shows the browning of the discs. Upon addition of L-cysteine the colour disappeared again showing that the discolouration is dependent on PPO.

[0133] This example demonstrates that chlorogenic acid is a suitable substrate for a substrate-based screening assay.

EXAMPLE 14

Substrate-based phenotypic screen diagnostic for the wound-induced discolouration for seeds

[0134] Seeds of lettuce and eggplant were incubated with 0, 2.5 and 5 mM L-DOPA. **Figure 19** shows that the colour reaction observed in the seeds is concentration dependent. The response is PPO-dependent because it can be inhibited with L-cysteine.

[0135] Germinated seeds of lettuce were incubated with L-DOPA. **Figure 20** shows that the seed coat and the root zone just behind the root tip show a colour reaction. This colour reaction can be used for screening seeds that show a reduced discolouration.

[0136] Lettuce seeds were incubated with 0, 100, 250, 500, 750 and 1000 mg/L catechol. **Figure 21** shows that seeds show a concentration dependent colour response. The response is PPO-dependent because it was found to be inhibited by L-cysteine.

Claims

1. Method for screening a population of plants or plant parts for the presence therein of individuals that show a reduced discolouration as compared to a control plant or plant part, which method comprises

   a) providing a population of plants or parts of the plants from the population;
   b) incubating the plants or plant parts having a wound surface created thereon in an aqueous environment to allow for discolouration to occur therein or thereon;
   c) observing the discolouration in or on the plants or plant parts;
   d) comparing the observed discolouration with the discolouration that is observed in the control plant or plant part to identify plants or plant parts that show no discolouration or a discolouration that is reduced as compared to the control plant or plant part,

wherein the plants or plant parts used in the screen are young plant material.

2. Method as claimed in claim 1, wherein the discolouration is wound-induced discolouration.

3. Method as claimed in claim 1 or 2, wherein the plants are vegetable plants, fruit-bearing plants or flowering plants.

4. Method as claimed in claim 3, wherein the plants are vegetable plants selected from lettuce, endive, witloof, potato, sweet potato, celeriac, mushrooms, artichoke and eggplant.

5. Method as claimed in claim 3, wherein the plants are fruit-bearing plants selected from apple, banana, avocado, peach, pear, apricot and mango.

6. Method as claimed in claim 3, wherein the plants are flowering plants selected from gerbera and chrysanthemum.

7. Method as claimed in any one of the claims 1-4, wherein the plants belong to the family *Asteraceae,* in particular to the genus *Lactuca*, more in particular to the species *Lactuca sativa.*

8. Method as claimed in any one of the claims 1-4, wherein the plants belong genus *Cichorium* and in particular to the species *Cichorium intybus* and *Cichorium endivia.*

9. Method as claimed in claim 1, 2 or 3, wherein the plant parts are selected from leafs, heads, shoots, roots, tubers, stems, flowers, fruits, seeds, germinated seeds, or pieces thereof and cells.

10. Method as claimed in claim 7 or 8, wherein the plant parts are leaf discs.

11. Method as claimed in claim 7 or 8, wherein the plant parts are discs from midrib tissue.

12. Method as claimed in any one of the claims 1-11, wherein the plant population is a population of mutant plants, a germplasm collection or a population of transgenic plants.

13. Method as claimed in claim 12, wherein the population of mutant plants is obtained by a mutagenesis treatment using chemicals and/or irradiation.

14. Method as claimed in any one of the claims 1-13, wherein the aqueous environment comprises wetted filter paper.

15. Method as claimed in any one of the claims 1-13, wherein the aqueous environment comprises water or a solution.

16. Method as claimed in claim 14 or 15, wherein the aqueous environment contains a compound selected from L- 3, 4- dihydroxyphenylalanine, chlorogenic acid, isochlorogenic acid, L- tyrosine and catechol.

17. Method as claimed in claim 14 or 15, wherein the aqueous environment contains a compound selected from the compounds listed in **Table 1**.

18. Method as claimed in any one of the claims 1-17, wherein the control plant is a plant of which a leaf disc when incubated between two sheets of wetted filter paper for 7 days at 5°C shows pink discolouration around the edges.

19. Method for screening a population of plants or plant parts for the presence therein of individuals that show a reduced discolouration as compared to a control plant or plant part, which method comprises:

a) providing a population of plants or parts of the plants from the population;
b) incubating the plants or plants parts with a substrate that can be converted into a coloured pigment to allow for discolouration to occur therein or thereon;
c) observing the discolouration in or on the plants or plant parts;
d) comparing the observed discolouration with the discolouration that is observed in the control plant or plant part to identify plants or plant parts that show no discolouration or a discolouration that is reduced as compared to the control plant or plant part, wherein the plant or plant parts to be screened are young plant material, seeds or germinated seeds.

20. Method as claimed in claim 19, wherein the substrate is selected from the compounds listed in **Table 1**.

21. Method as claimed in claim 20, wherein the compound is L-DOPA.

22. Method as claimed in claim 19, 20 or 21, wherein the plant or plant part is wounded before incubation with the substrate and the discolouration of the wound or near the wound is observed.

Patentansprüche

1. Verfahren zum Screening eines Bestandes von Pflanzen oder Pflanzenteilen auf das Vorliegen in diesen von Individuen, die eine verminderte Verfärbung im Vergleich zu einer Kontrollpflanze oder einem Kontrollpflanzenteil aufweisen, wobei das Verfahren folgendes umfasst:

a) Bereitstellen eines Bestandes von Pflanzen oder Teilen der Pflanzen aus dem Bestand;
b) Bebrüten der Pflanzen oder Pflanzenteile mit einer darauf erzeugten Wundoberfläche in einer wässrigen Umgebung, um das Auftreten von Verfärbung in dieser oder auf dieser zu ermöglichen;
c) Beobachten der Verfärbung in oder auf den Pflanzen oder Pflanzenteilen;
d) Vergleichen der beobachteten Verfärbung mit der Verfärbung, die bei der Kontrollpflanze oder dem Kontrollpflanzenteil beobachtet wird, um Pflanzen oder Pflanzenteile zu bestimmen, die keine Verfärbung oder eine Verfärbung zeigen, die im Vergleich zur Kontrollpflanze oder zu dem Kontrollpflanzenteil verringert ist,

wobei die in dem Screening verwendeten Pflanzen oder Pflanzenteile aus jungem Pflanzenmaterial bestehen.

2. Verfahren gemäß Anspruch 1, wobei die Verfärbung eine wundinduzierte Verfärbung darstellt.

3. Verfahren gemäß Anspruch 1 oder 2, wobei die Pflanzen Gemüsepflanzen, Obstpflanzen oder Blütenpflanzen sind.

4. Verfahren gemäß Anspruch 3, wobei die Pflanzen Gemüsepflanzen ausgewählt aus Kopfsalat, Endiviensalat, Chicoree, Kartoffel, Süßkartoffel, Sellerie, Pilzen, Artischocke und Aubergine sind.

5. Verfahren gemäß Anspruch 3, wobei die Pflanzen Obstpflanzen ausgewählt aus Apfel, Banane, Avocado, Pfirsich, Birne, Aprikose und Mango sind.

6. Verfahren gemäß Anspruch 3, wobei die Pflanzen Blütenpflanzen ausgewählt aus Gerbera und Chrysantheme sind.

7. Verfahren gemäß einem der Ansprüche 1 bis 4, wobei die Pflanzen zur Familie der *Asteraceae*, insbesondere zur Gattung der *Lactuca* und besonders zur Species der *Lactuca sativa* gehören.

8. Verfahren gemäß einem der Ansprüche 1 bis 4, wobei die Pflanzen zur Gattung *Cichorium* und insbesondere zur Species *Cichorium intybus* und *Cichorium endivia* gehören.

9. Verfahren gemäß Anspruch 1, 2 oder 3, wobei die Pflanzenteile ausgewählt sind aus Blättern, Köpfen, Schösslingen, Wurzeln, Knollen, Stielen, Blüten, Früchten, Samen, gekeimten Samen oder Teilen von diesen sowie Zellen.

10. Verfahren gemäß Anspruch 7 oder 8, wobei die Pflanzenteile Blattscheiben sind.

11. Verfahren gemäß Anspruch 7 oder 8, wobei die Pflanzenteile Scheiben aus Hauptadergewebe darstellen.

12. Verfahren gemäß einem der Ansprüche 1 bis 11, wobei der Pflanzenbestand aus einem Bestand von Mutationspflanzen, einer Ansammlung von Keimplasma oder einem Bestand von transgenen Pflanzen besteht.

13. Verfahren gemäß Anspruch 12, wobei der Bestand von Mutationspflanzen durch eine Mutagenesebehandlung unter Einsatz von Chemikalien und/oder Bestrahlung gewonnen wird.

14. Verfahren gemäß einem der Ansprüche 1 bis 13, wobei die wässrige Umgebung befeuchtetes Filterpapier umfasst.

15. Verfahren gemäß einem der Ansprüche 1 bis 13, wobei die wässrige Umgebung Wasser oder eine Lösung umfasst.

16. Verfahren gemäß Anspruch 14 oder 15, wobei die wässrige Lösung eine Verbindung ausgewählt aus L-3, 4-Dihydroxyphenylalanin, Chlorogensäure, Isochlorogensäure, L-Tyrosin und Katechin enthält.

17. Verfahren gemäß Anspruch 14 oder 15, wobei die wässrige Umgebung eine Verbindung ausgewählt aus den in **Tabelle 1** aufgeführten Verbindungen enthält.

18. Verfahren gemäß einem der Ansprüche 1 bis 17, wobei die Kontrollpflanze eine Pflanze ist, aus der eine Blattscheibe nach dem Bebrüten zwischen zwei Bögen von befeuchtetem Filterpapier über 7 Tage bei 5°C eine rosarote Verfärbung um die Kanten herum zeigt.

19. Verfahren zum Screening eines Bestandes von Pflanzen oder Pflanzenteilen auf das Vorliegen in diesen von Individuen, die eine verringerte Verfärbung im Vergleich zu einer Kontrollpflanze oder einem Kontrollpflanzenteil zeigen, wobei das Verfahren folgendes umfasst:

    a) Bereitstellen eines Bestandes von Pflanzen oder von Teilen der Pflanzen aus dem Bestand;
    b) Bebrüten der Pflanzen oder Pflanzenteile mit einem Substrat, das in ein farbiges Pigment umgewandelt werden kann, um das Auftreten von Verfärbung in oder auf diesen zu ermöglichen;
    c) Beobachten der Verfärbung in oder auf den Pflanzen oder Pflanzenteilen;
    d) Vergleichen der beobachteten Verfärbung mit der Verfärbung, die in der Kontrollpflanze oder dem Kontrollpflanzenteil beobachtet wird, um Pflanzen oder Pflanzenteile zu bestimmen, die keine Verfärbung oder eine Verfärbung zeigen, die im Vergleich zu der Kontrollpflanze oder dem Kontrollpflanzenteil verringert ist, wobei die dem Screening zu unterziehende Pflanze oder der Pflanzenteil aus jungem Pflanzenmaterial, Samen oder gekeimten Samen besteht.

20. Verfahren gemäß Anspruch 19, wobei das Substrat aus den in **Tabelle 1** aufgeführten Verbindungen ausgewählt ist.

21. Verfahren gemäß Anspruch 20, wobei die Verbindung L-DOPA ist.

22. Verfahren gemäß Anspruch 19, 20 oder 21, wobei die Pflanze oder der Pflanzenteil vor dem Bebrüten mit dem Substrat verwundet wird und die Verfärbung der Wunde oder nahe der Wunde beobachtet wird.

Revendications

1. Procédé de criblage d'une population de plantes ou de parties de plantes pour identifier la présence de sujets qui présentent une décoloration réduite comparativement à une plante ou partie de plante témoin, le procédé comprenant

    a) l'utilisation d'une population de plantes ou de parties de plantes appartenant à cette population ;
    b) l'incubation des plantes ou parties de plantes présentant une surface lésée créée dans un environnement aqueux pour laisser la décoloration se produire dans ou sur les plantes ou parties de plantes ;
    c) l'observation de la décoloration dans ou sur les plantes ou parties de plantes ;
    d) la comparaison de la décoloration observée à la décoloration qui est observée chez la plante ou partie de plante témoin pour identifier les plantes ou parties de plantes qui ne présentent aucune décoloration ou présentent une décoloration qui est réduite comparativement à la plante ou partie de plante témoin,

    dans lequel les plantes ou parties de plantes utilisées dans le criblage sont un matériel végétal jeune.

2. Procédé selon la revendication1, dans lequel la décoloration est une décoloration induite par une lésion.

3. Procédé selon la revendication 1 ou 2, dans lequel les plantes sont des légumes, des plantes à fruits ou des plantes à fleurs.

4. Procédé selon la revendication 3, dans lequel les plantes sont des légumes choisis parmi la laitue, l'endive, le chicon, la pomme de terre, la patate douce, le célerirave, les champignons, l'artichaut et l'aubergine.

5. Procédé selon la revendication 3, dans lequel les plantes sont des plantes à fruits choisis parmi la pomme, la banane, l'avocat, la pêche, la poire, l'abricot et la mangue.

6. Procédé selon la revendication 3, dans lequel les plantes sont des plantes à fleurs choisies parmi le gerbera et le chrysanthème.

7. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel les plantes appartiennent à la famille des *Asteraceae,* en particulier au genre *Lactuca,* plus particulièrement à l'espèce *Lactuca sativa.*

8. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel les plantes appartiennent au genre *Cichorium* et en particulier aux espèces *Cichorium intybus* et *Cichorium endivia.*

9. Procédé selon la revendication 1, 2 ou 3, dans lequel les parties de plantes sont choisies parmi les feuilles, les têtes, les pousses, les racines, les tubercules, les tiges, les fleurs, les fruits, les graines, les graines germées, ou des fragments de ceux-ci et des cellules.

10. Procédé selon la revendication 7 ou 8, dans lequel les parties de plantes sont des disques de feuilles.

11. Procédé selon la revendication 7 ou 8, dans lequel les parties de plantes sont des disques de tissu de nervure centrale.

12. Procédé selon l'une quelconque des revendications 1 à 11, dans lequel la population de plantes est une population de plantes mutantes, une collection de germoplasmes ou une population de plantes transgéniques.

13. Procédé selon la revendication 12, dans lequel la population de plantes mutantes est obtenue par un traitement de mutagenèse à base d'agents chimiques et/ou d'irradiation.

14. Procédé selon l'une quelconque des revendications 1 à 13, dans lequel l'environnement aqueux comprend un papier filtre humidifié.

15. Procédé selon l'une quelconque des revendications 1 à 13, dans lequel l'environnement aqueux comprend de l'eau ou une solution.

16. Procédé selon la revendication 14 ou 15, dans lequel l'environnement aqueux contient un composé choisi parmi la L- 3, 4- dihydroxyphénylalanine, l'acide chlorogène, l'acide isochlorogène, la L- tyrosine et le catéchol.

17. Procédé selon la revendication 14 ou 15, dans lequel l'environnement aqueux contient un composé choisi parmi les composés indiqués dans le **Tableau 1**.

18. Procédé selon l'une quelconque des revendications 1 à 17, dans lequel la plante témoin est une plante dont un disque de feuille incubé entre deux feuilles de papier filtre humidifiées pendant 7 jours à 5 °C présente une décoloration rose le long des bords.

19. Procédé de criblage d'une population de plantes ou de parties de plantes pour identifier la présence de sujets qui présentent une décoloration réduite comparativement à une plante ou partie de plante témoin, le procédé comprenant :

a) l'utilisation d'une population de plantes ou de parties de plantes appartenant à cette population ;
b) l'incubation des plantes ou parties de plantes avec un substrat qui peut être converti en pigment coloré pour laisser la décoloration se produire dans ou sur les plantes ou parties de plantes ;
c) l'observation de la décoloration dans ou sur les plantes ou parties de plantes ;
d) la comparaison de la décoloration observée à la décoloration qui est observée chez la plante ou partie de plante témoin pour identifier les plantes ou parties de plantes qui ne présentent aucune décoloration ou présentent une décoloration qui est réduite comparativement à la plante ou partie de plante témoin,

dans lequel la plante ou les parties de plante à cribler sont un matériel végétal jeune, des graines ou des graines germées.

20. Procédé selon la revendication 19, dans lequel le substrat est choisi parmi les composés indiqués dans le **Tableau 1**.

21. Procédé selon la revendication 20, dans lequel le composé est la L-DOPA.

22. Procédé selon la revendication 19, 20 ou 21, dans lequel la plante ou partie de plante est blessée avant incubation avec le substrat et la décoloration de la lésion ou à proximité de la lésion est observée.

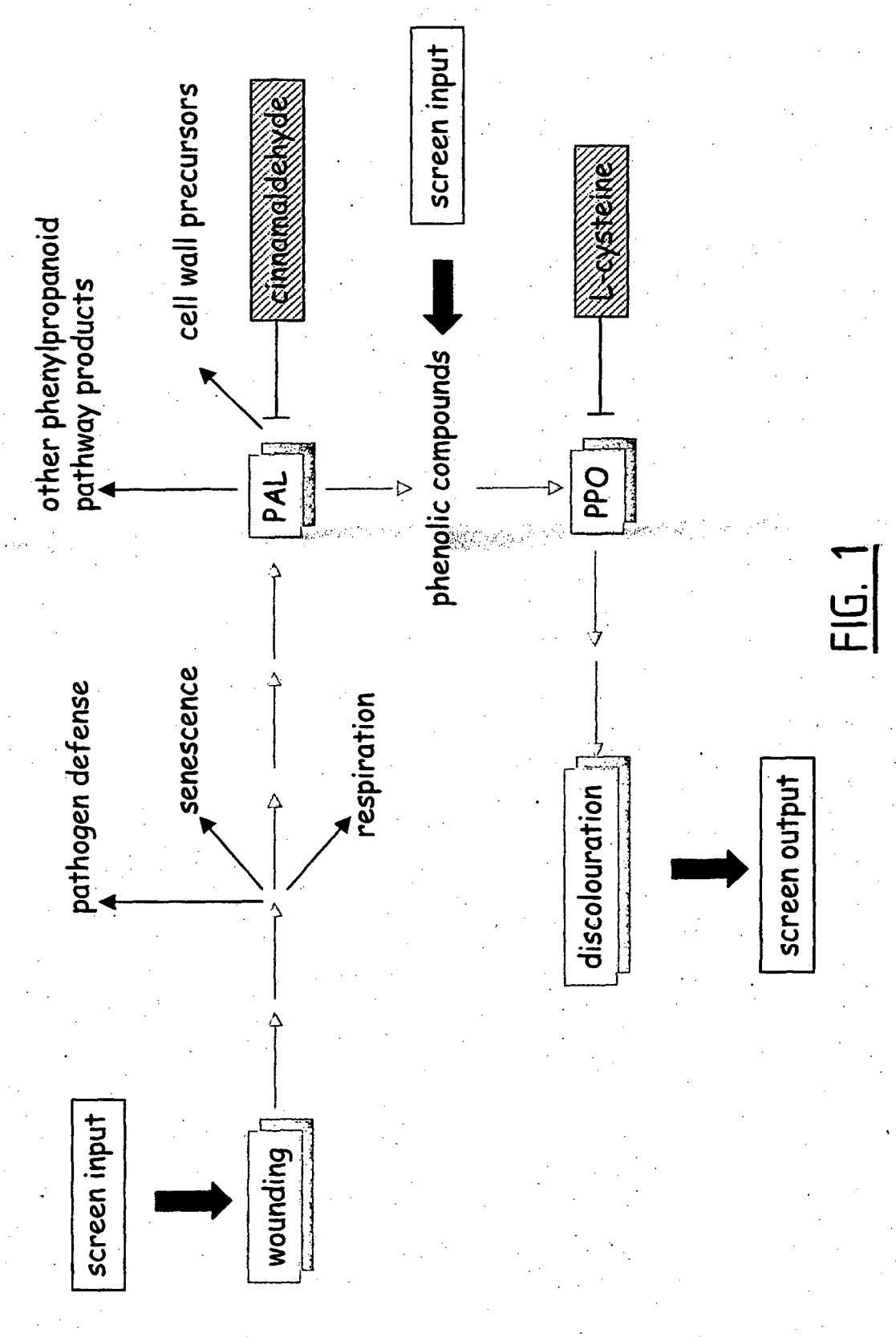
screen input
wounding
pathogen defense
senescence
respiration
other phenylpropanoid pathway products
cell wall precursors
cinnamaldehyde
PAL
phenolic compounds
screen input
L-cysteine
PPO
discolouration
screen output

FIG. 1

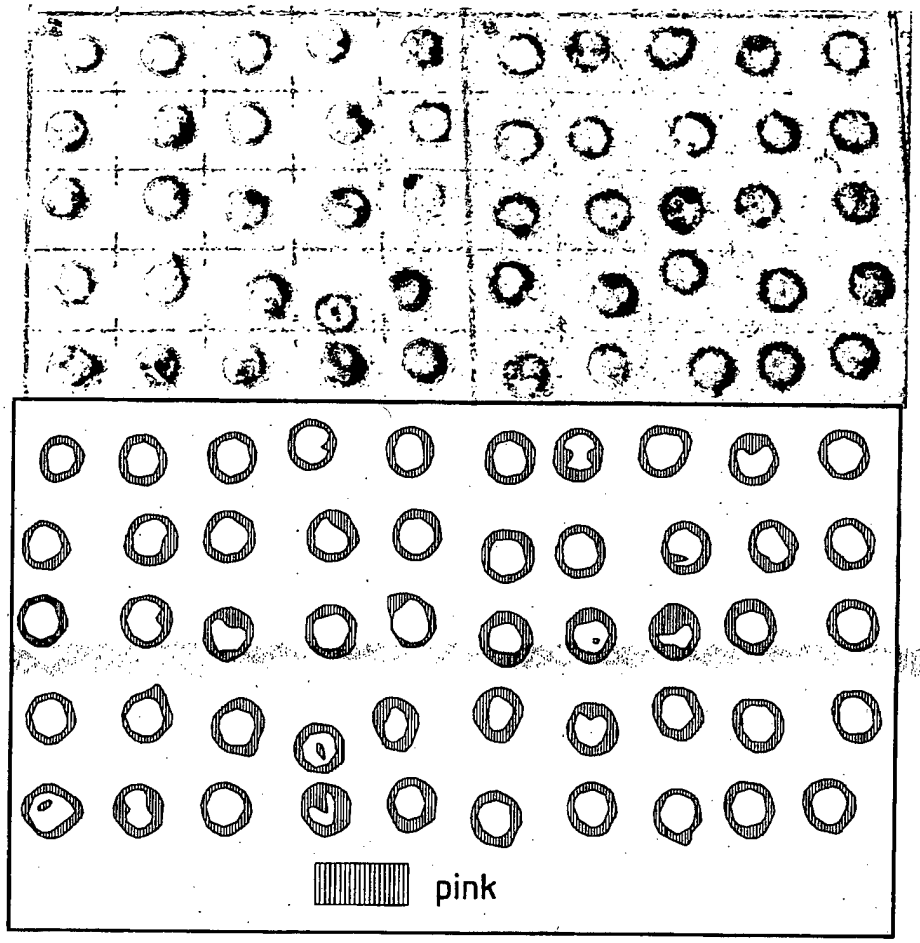

FIG. 2

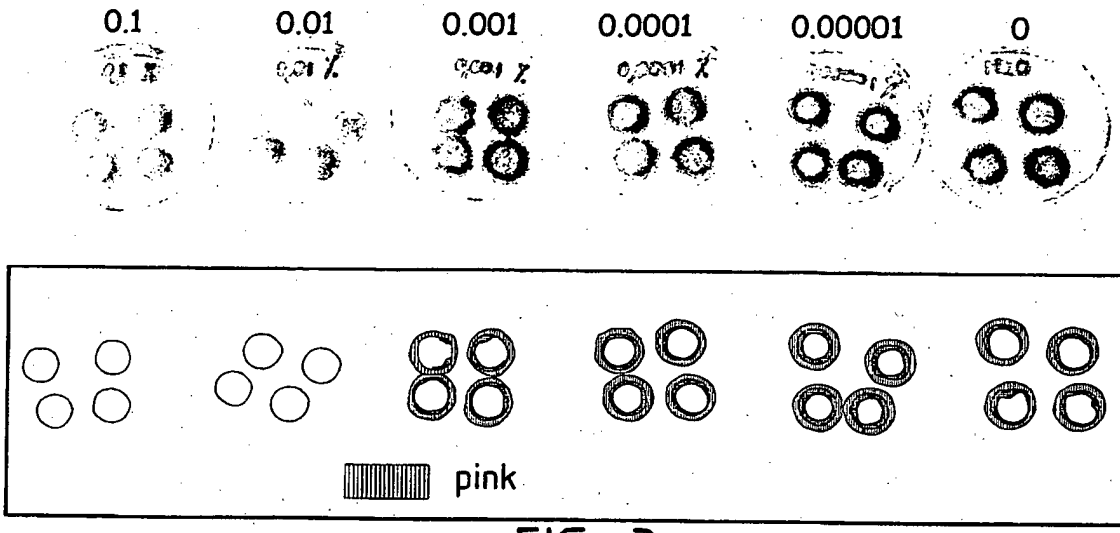

FIG. 3

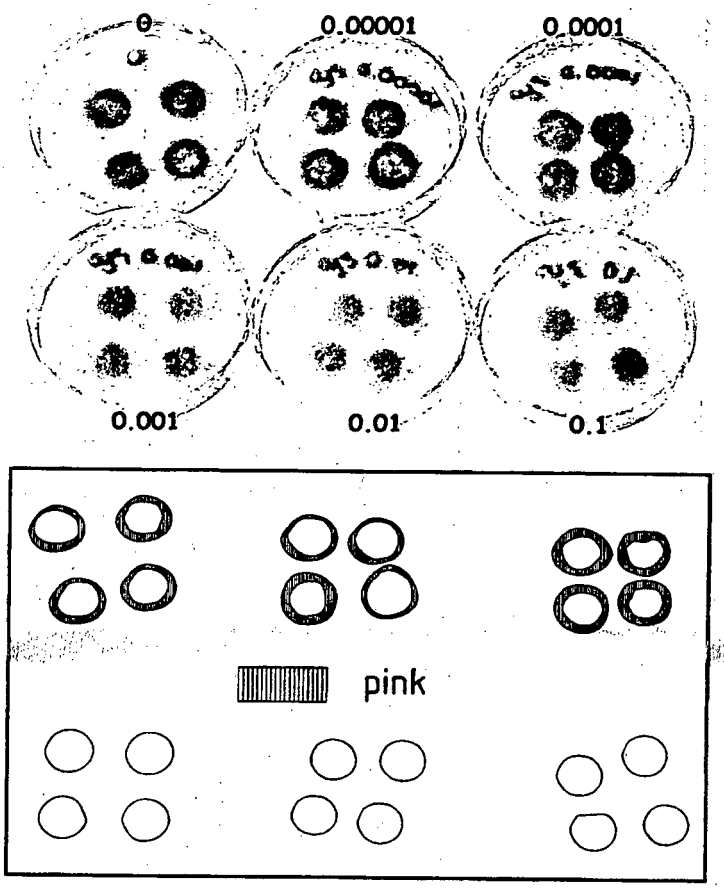

FIG. 4

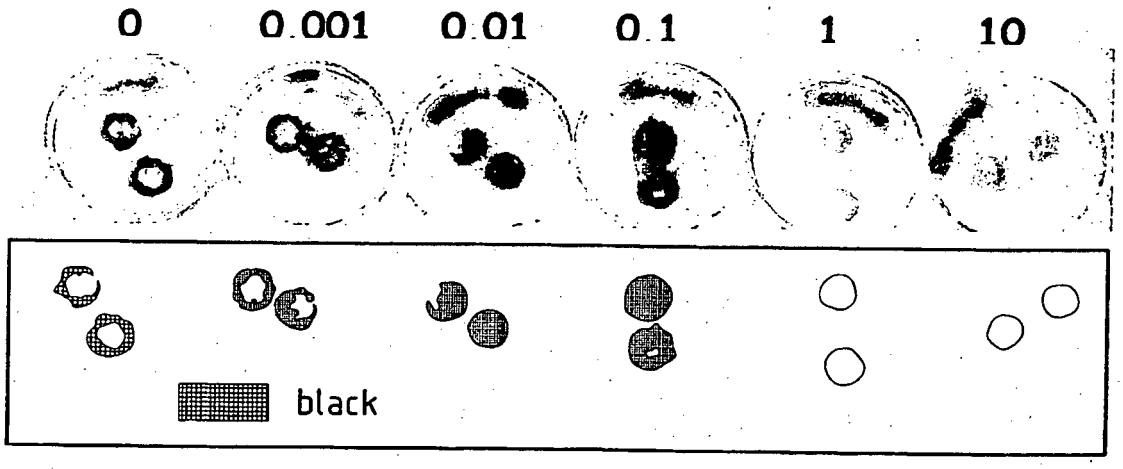

FIG. 5

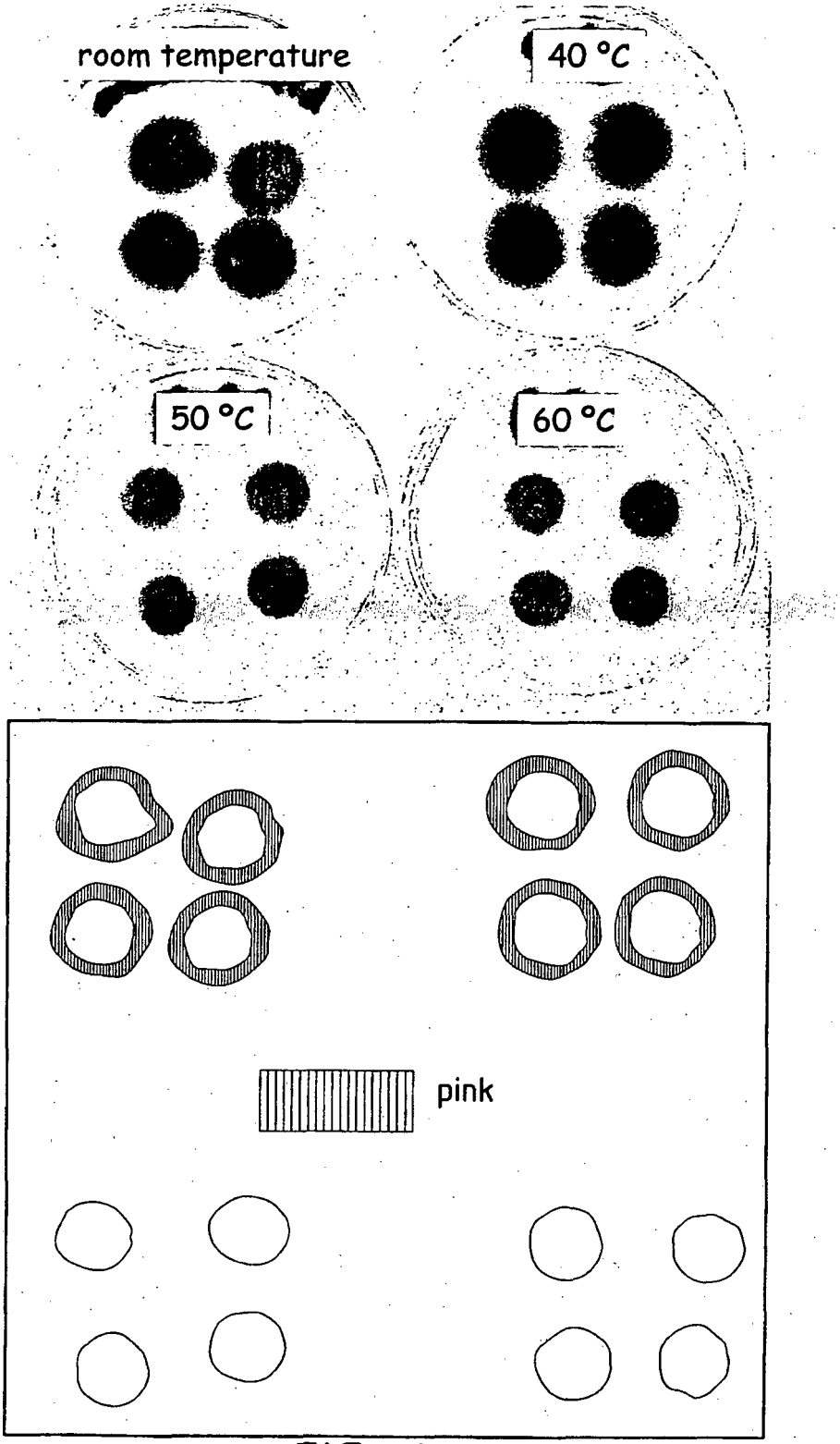

FIG. 6

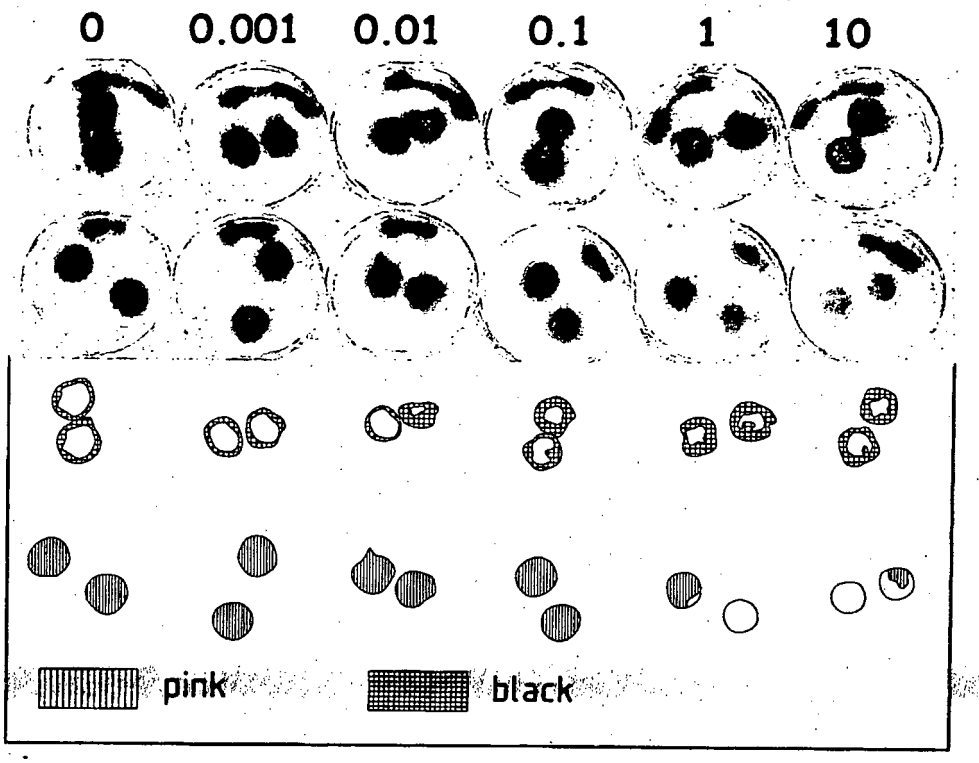

FIG. 7

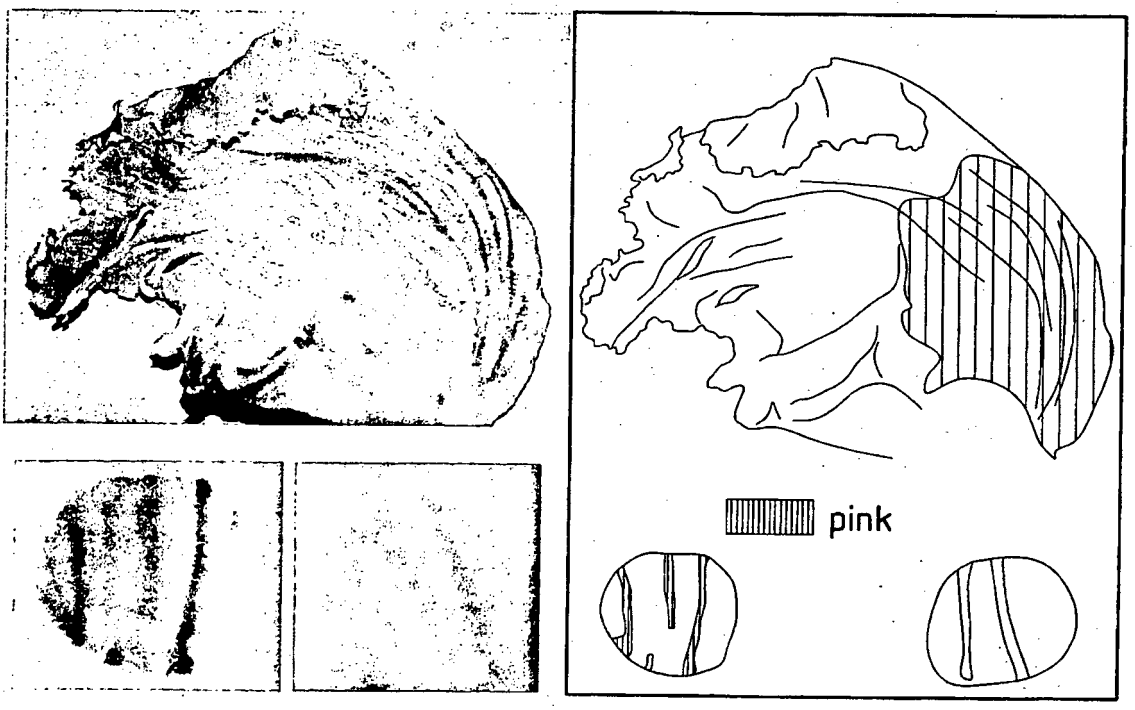

FIG. 8

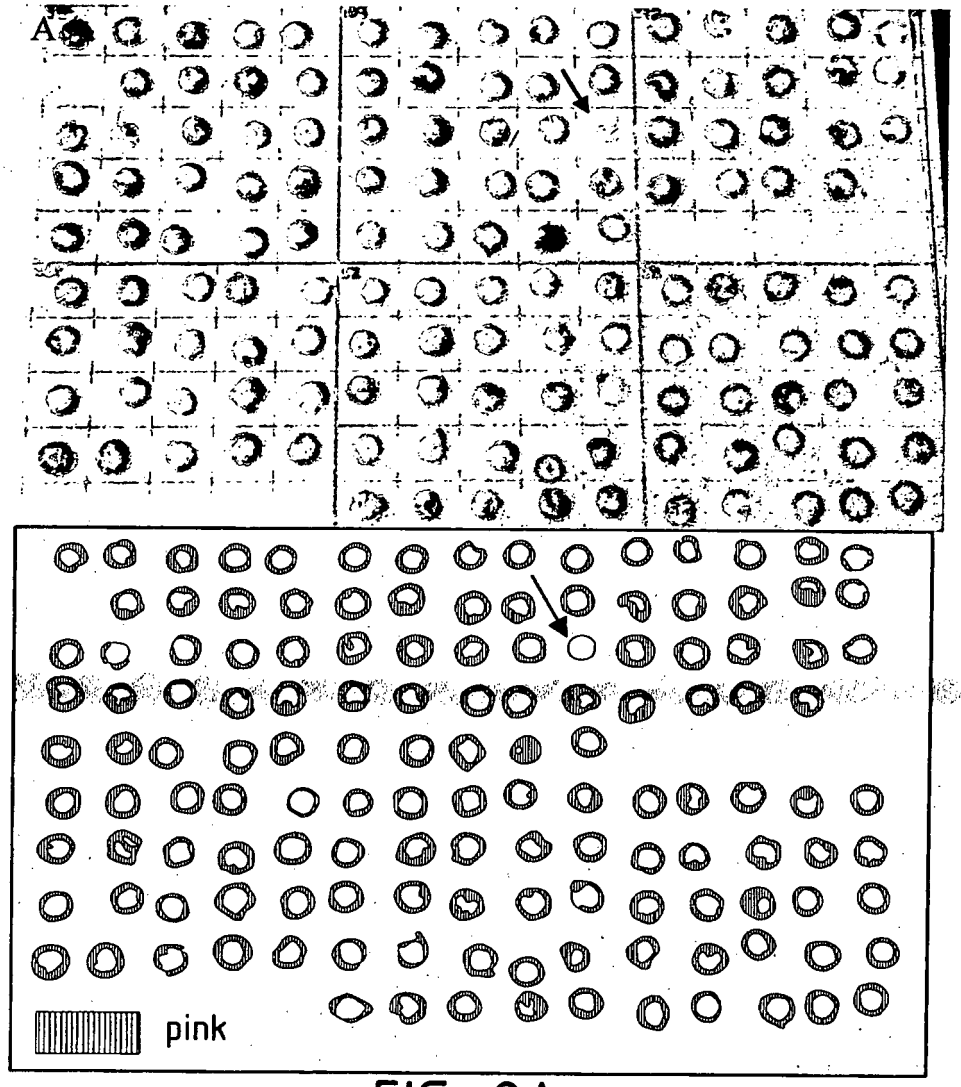

FIG. 9A

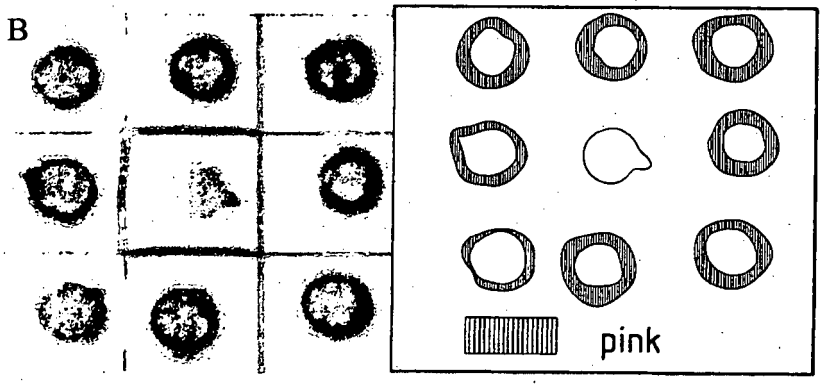

FIG. 9B

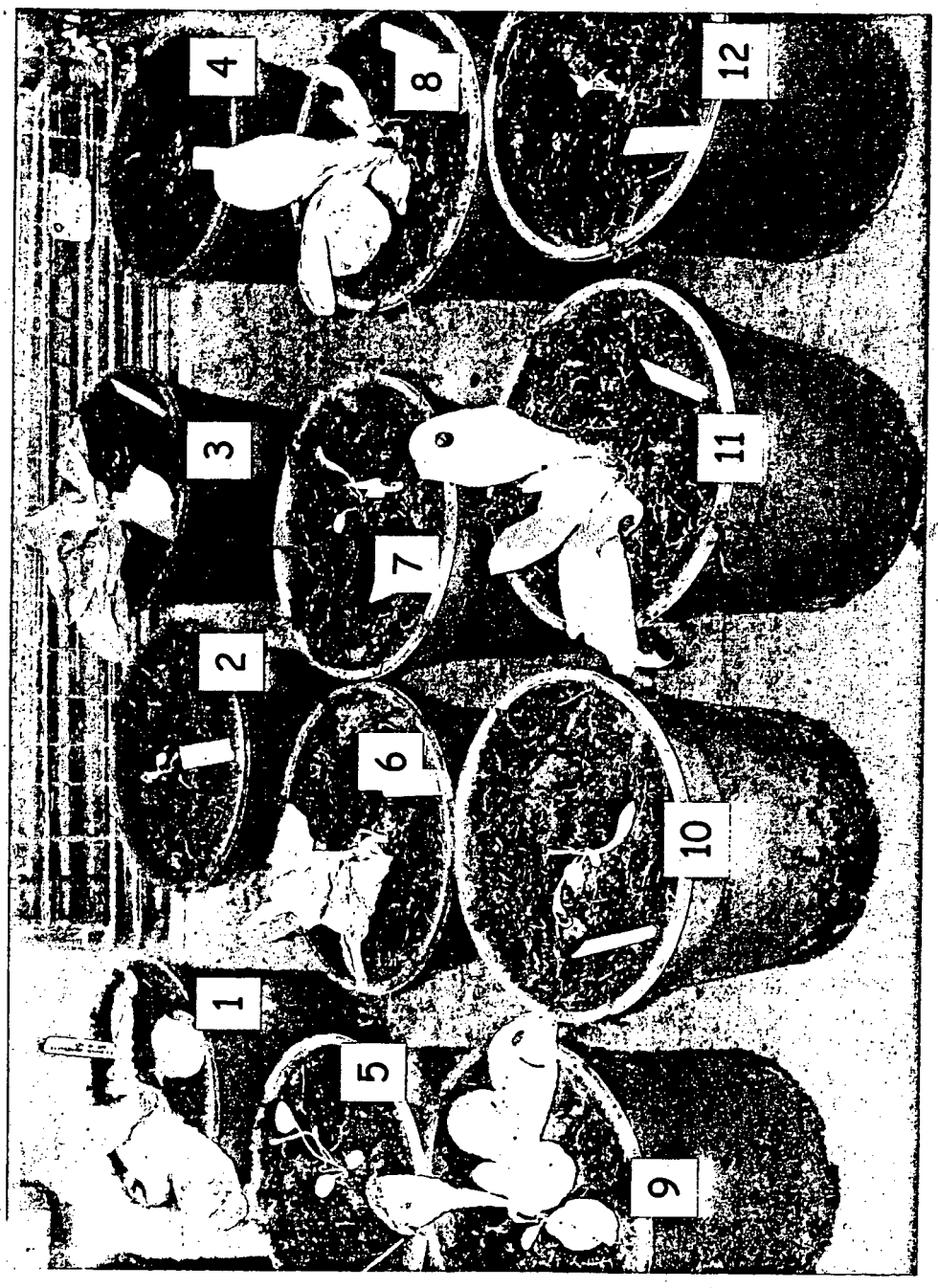

FIG. 10

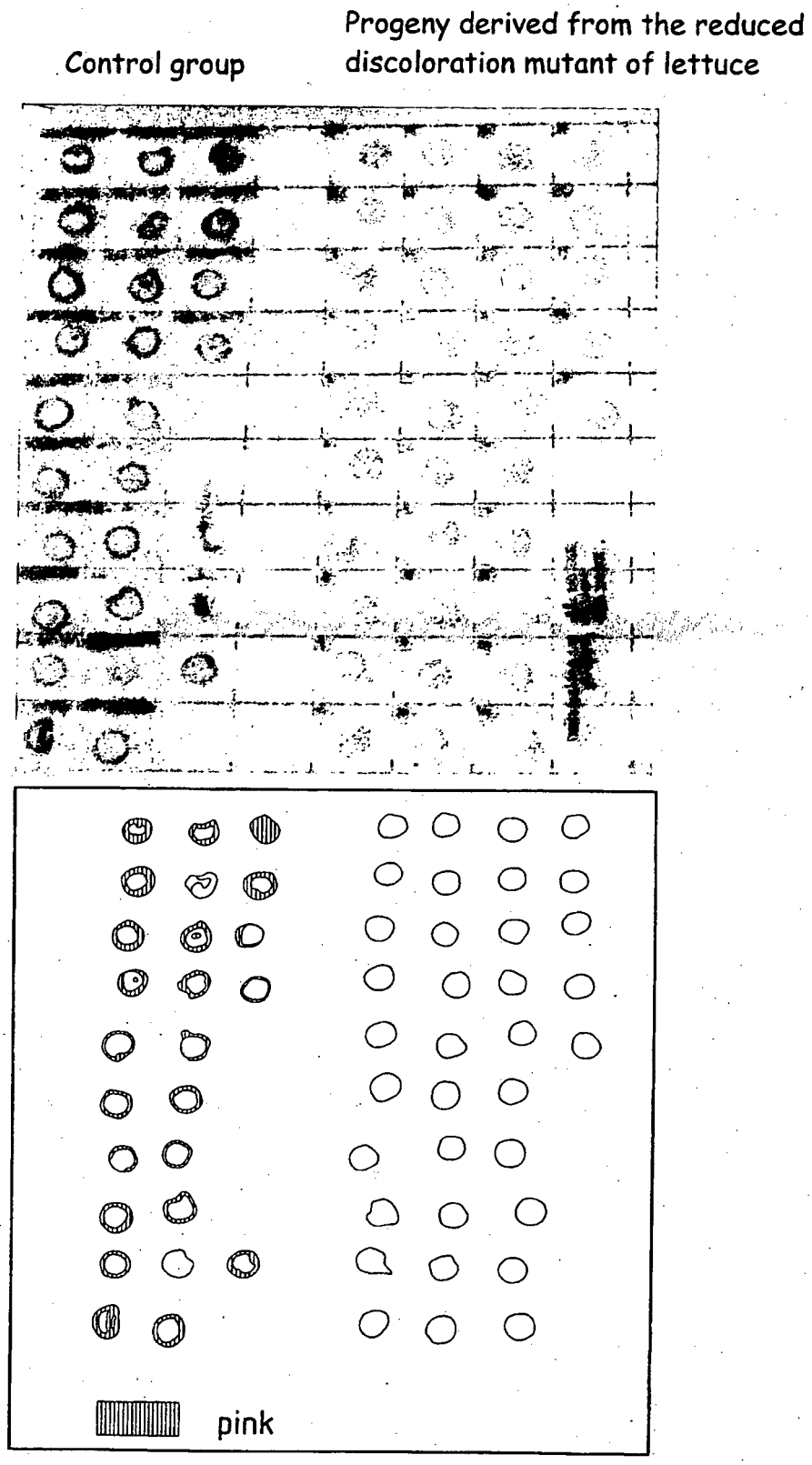
Control group
Progeny derived from the reduced discoloration mutant of lettuce
pink

FIG. 11

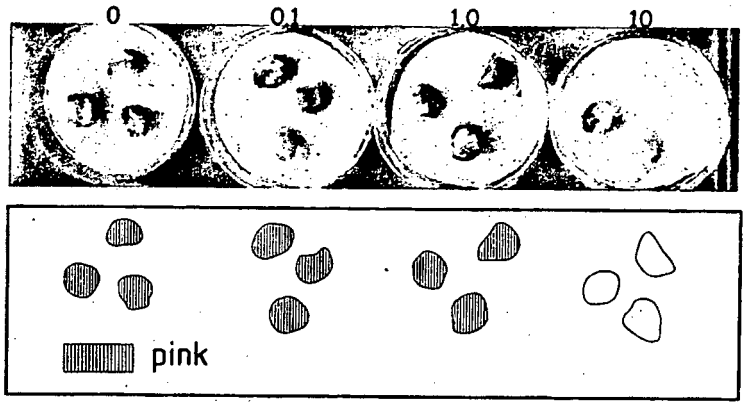

FIG. 12

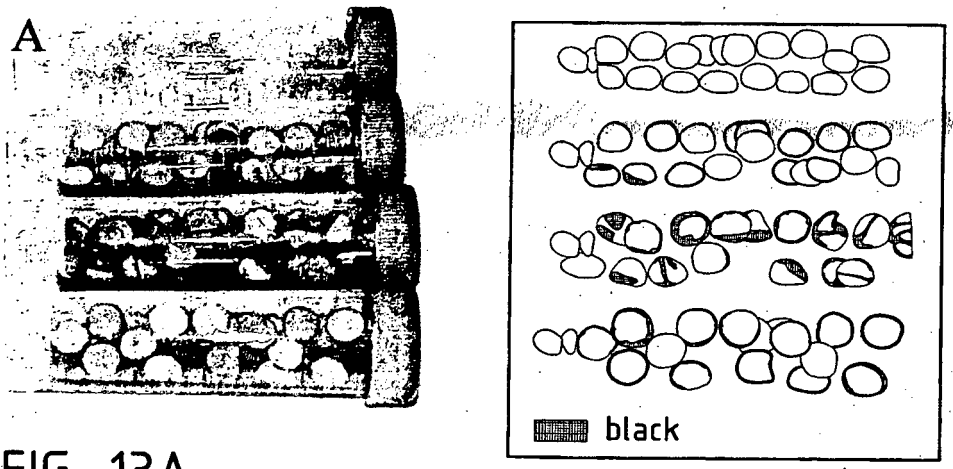

FIG. 13A

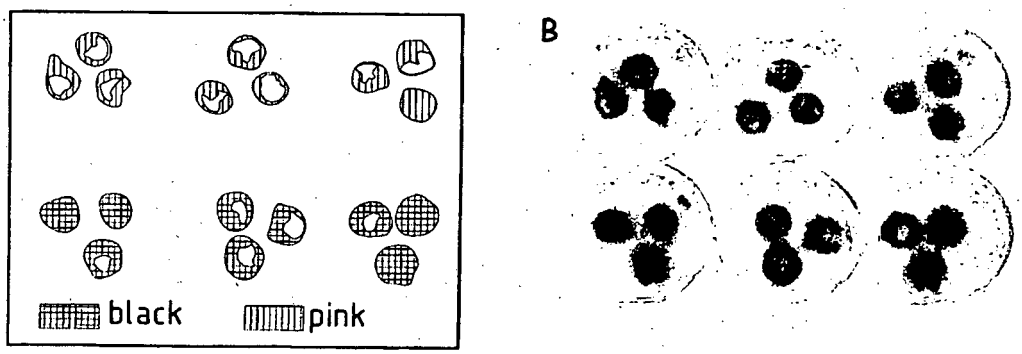

FIG. 13B

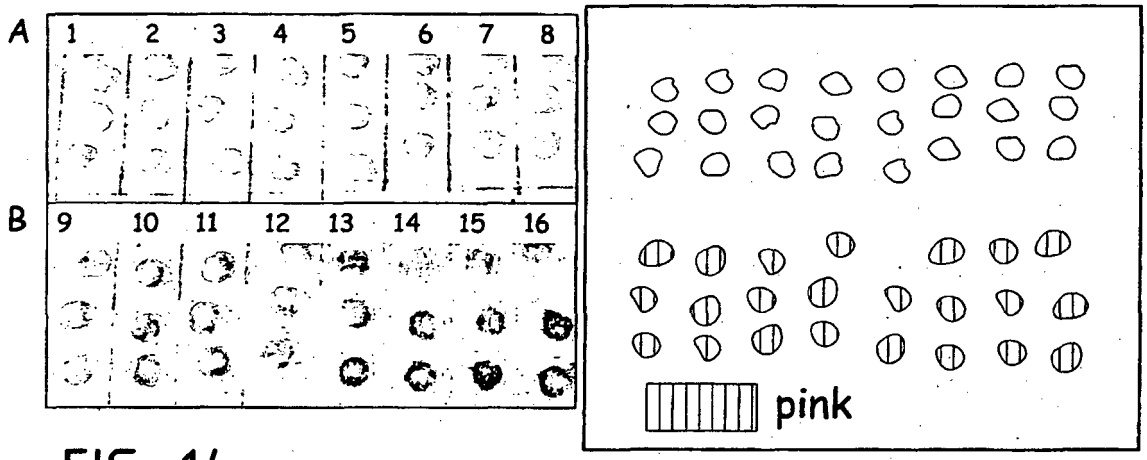
A
1
2
3
4
5
6
7
8
B
9
10
11
12
13
14
15
16
pink

FIG. 14

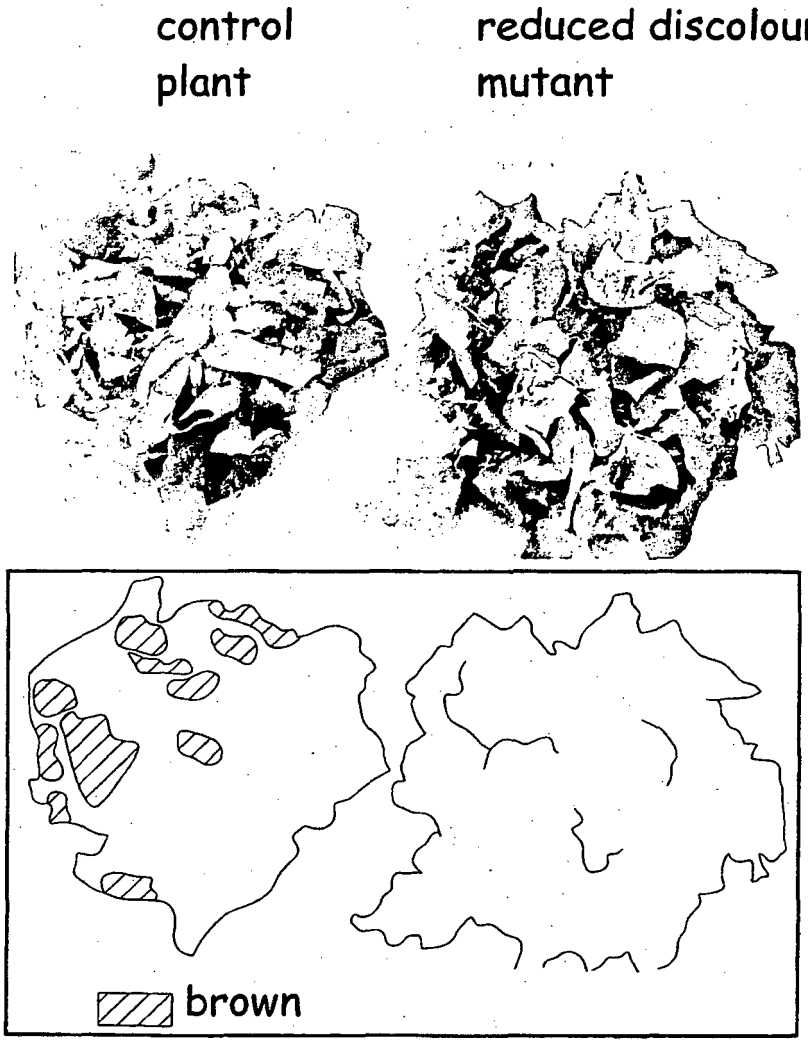
control plant
reduced discolouration mutant
brown

FIG. 15

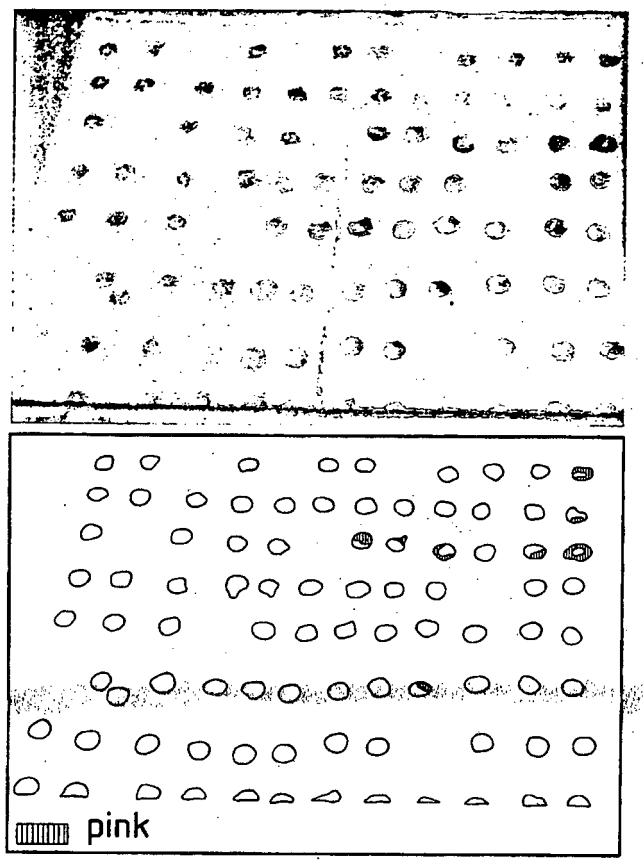

FIG. 16

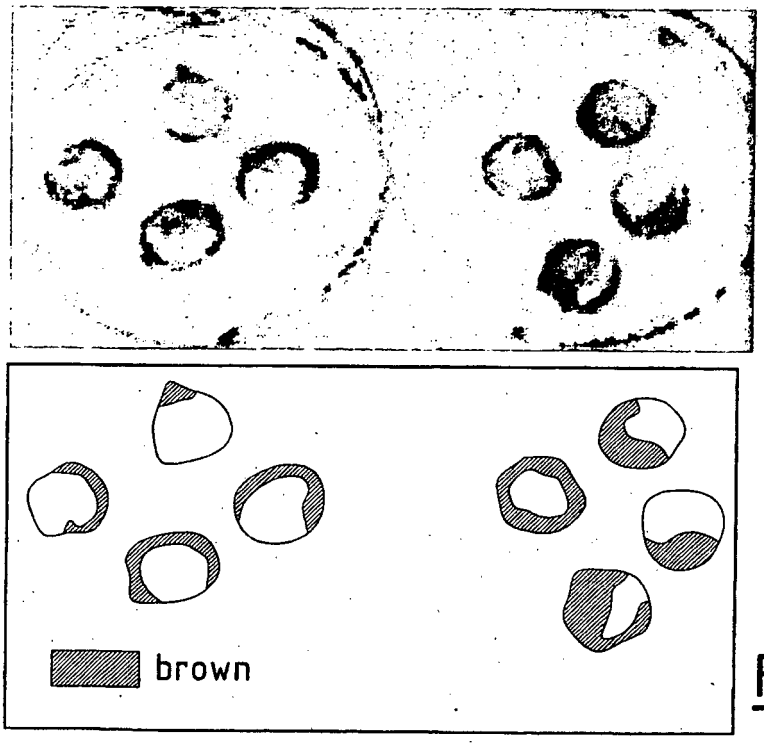

FIG. 17

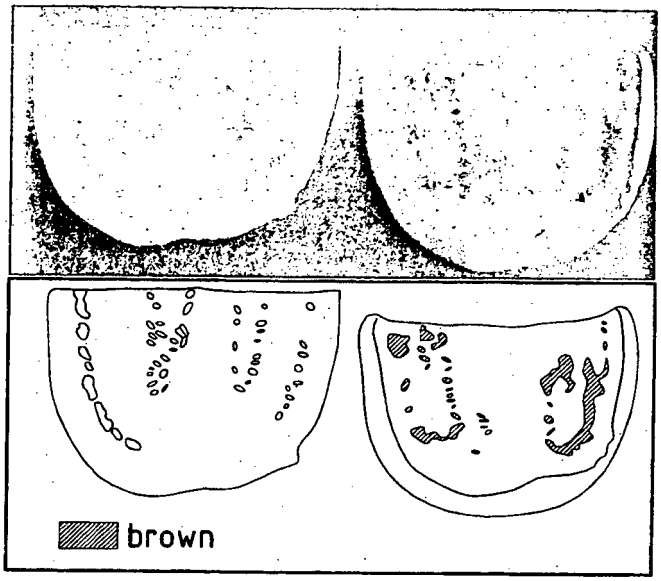

FIG. 18

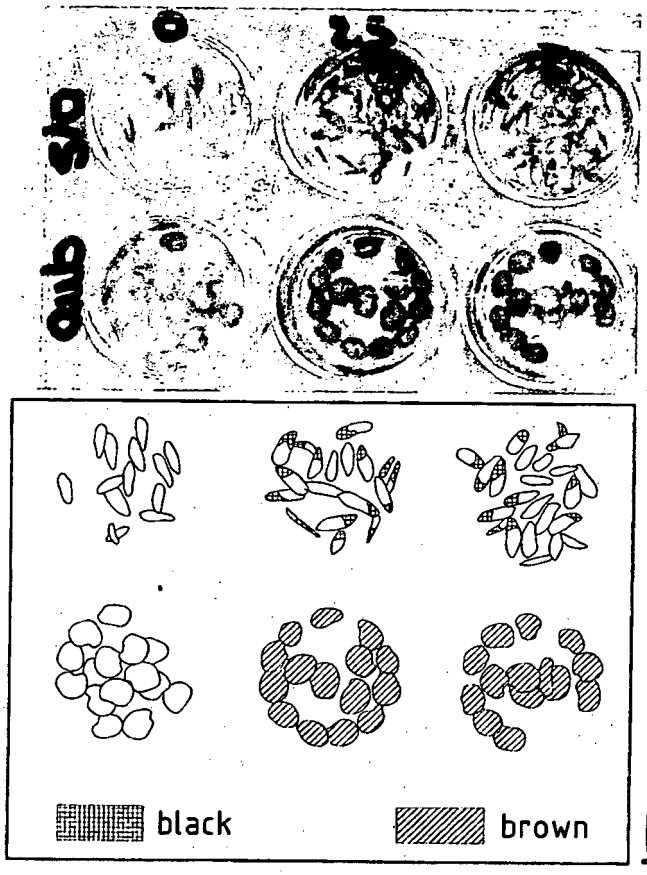

FIG. 19

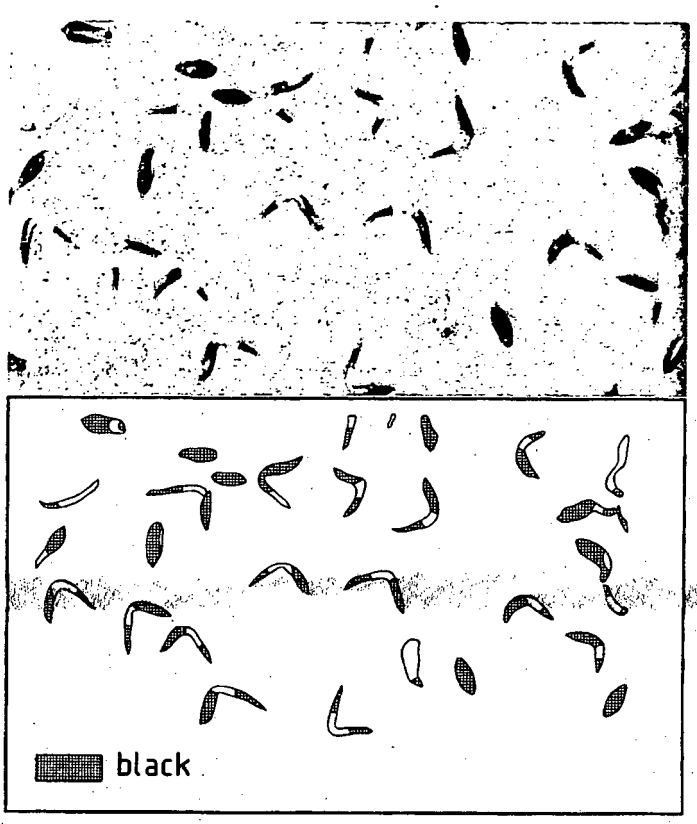

FIG. 20

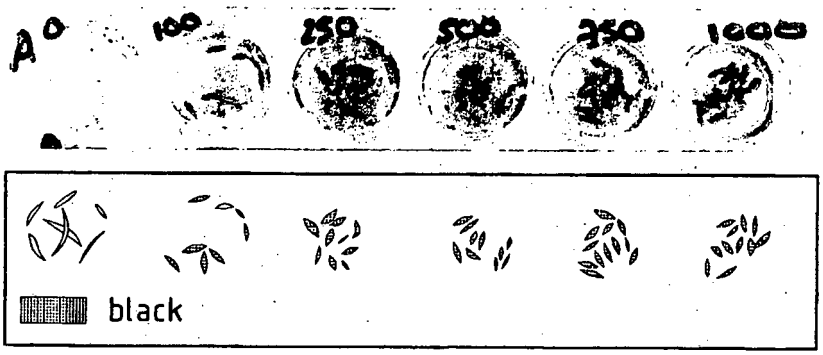

FIG. 21

REFERENCES CITED IN THE DESCRIPTION

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