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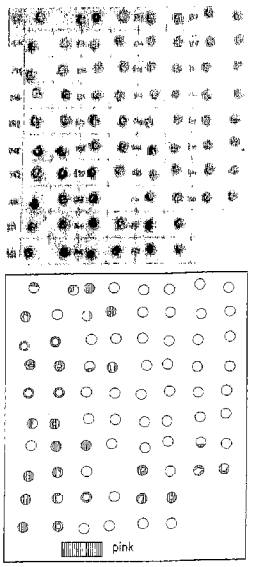
(54) Titre : PROCEDE D'IDENTIFICATION D'UNE MATIERE VEGETALE QUI PRESENTE UNE ALTERATION DE COULEUR, PLANTES AINSI IDENTIFIEES, ET UTILISATION DES PLANTES IDENTIFIEES POUR LA PRODUCTION DE PLANTES A RECOLTE INDUSTRIELLE PRESENTANT UNE ALTERATION DE COULEUR

(54) Title: METHOD FOR IDENTIFYING PLANT MATERIAL THAT SHOWS A REDUCED DISCOLOURATION, PLANTS THUS IDENTIFIED AND USE OF THE IDENTIFIED PLANTS FOR THE PRODUCTION OF COMMERCIAL CROP PLANTS SHOWING A REDUCED DISCOLOURATION



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The invention relates to a method for testing a plant or plant part for exhibiting a reduced or absent wound-induced surface discolouration as compared to a control plant or plant part, which method comprises providing a plant or plant part that is obtained from a plant population that shows genetic variation, in particular a gene bank; optionally creating a wound surface on the plant or plant part; incubating the plant or plant part or the wound surfaces created thereon to allow for discolouration to occur therein or thereon; observing the discolouration in or on the plants or plant parts; comparing the observed discolouration with the discolouration that is observed in the control plant or plant part to assess whether the plant or plant part shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part; and identifying a plant or plant part that shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part as a plant that harbours the reduced wound-induced surface discolouration trait.

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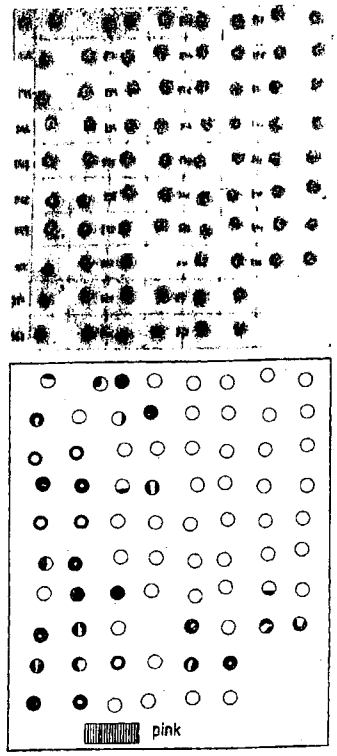

FIG. 1

(57) Abstract: The invention relates to a method for testing a plant or plant part for exhibiting a reduced or absent wound-induced surface discolouration as compared to a control plant or plant part, which method comprises providing a plant or plant part that is obtained from a plant population that shows genetic variation, in particular a gene bank; optionally creating a wound surface on the plant or plant part; incubating the plant or plant part or the wound surfaces created thereon to allow for discolouration to occur therein or thereon; observing the discolouration in or on the plants or plant parts; comparing the observed discolouration with the discolouration that is observed in the control plant or plant part to assess whether the plant or plant part shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part; and identifying a plant or plant part that shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part as a plant that harbours the reduced wound-induced surface discolouration trait.

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METHOD FOR IDENTIFYING PLANT MATERIAL THAT SHOWS A REDUCED DISCOLOURATION, PLANTS THUS IDENTIFIED AND USE OF THE IDENTIFIED PLANTS FOR THE PRODUCTION OF COMMERCIAL CROP PLANTS SHOWING A REDUCED DISCOLOURATION

Field of the invention

The present invention relates to a method for identifying plant material for the presence therein of material that shows a reduced discolouration, in particular wound-induced surface discolouration, as compared to control plant material.

The invention further relates to plant material thus identified, to the use thereof in breeding and to plants obtained thereby.

Background of the invention

Harvesting and subsequent processing, packaging and storage of produce evokes a strong response of the plant material which in general leads to a fast reduction of the quality of the end product. Quality in this respect refers to several attributes like colour, taste, smell, crispness which is perceived by the consumer as freshness.

The response of produce towards processing can be of a combined physical, biochemical and physiological nature resulting in wilting, discolouration and senescence. In order to improve the quality of processed produce it will become increasingly important to develop plant varieties which show reduced levels of deterioration as a consequence of processing.

Lettuce is currently one of the fresh products which is increasingly offered to the consumer in a processed i.e. cut, washed, packaged form. This ready-to-eat product contributes to the convenience of the consumer but in

addition allows the development of new products like mixes of different leafy or other types of vegetables.

The most important problem which occurs during lettuce processing and storage is the wound surface discolouration. This disorder manifests as a pink or brown colouration at the wound surface of the cut leaves. Especially the mid rib sections can show a strong staining in this respect. This negative quality trait is the most important trait to be controlled during processing, as packaged lettuce with a pink or brown discolouration is perceived by the consumer as a deteriorated product.

Although post-harvest treatments, like packaging under a controlled atmosphere, prevent discolouration and have improved the quality of fresh cut, packaged lettuce, a genetic solution is preferred. The reasons are that despite these treatments, a substantial part of the product is still lost due to causes such as damaged packages. Also shelf life after opening the package by the end user is very short. And, importantly, there is significant cost involved.

Therefore, breeding for lettuce plants which have improved processing traits, in particular reduced surface discolouration, is of utmost importance for the lettuce processing industry. The same applies to the processing of other plant material, such as witloof and eggplant.

It is thus the object of the invention to enable the fast and efficient identification of plant material that has a reduced surface discolouration for use thereof in the production of crop plants that are less prone to post-harvest deterioration.

Summary of the invention

The wound-induced discolouration process is a means of a plant to defend itself against damage via wound surfaces

caused by pathogens, pests or physical factors (wind, rain, drought, cold, heat, etc.). It is therefore expected that natural variation for wound-induced discolouration is absent due to natural selection. Plants that are not able to defend themselves against external threats are weaker and have therefore less chances of surviving such threats.

Nevertheless, it was surprisingly found according to the invention that gene bank material contains significant variation in discolouration, in particular wound-induced discolouration. The inventors were able to demonstrate this by using a method for testing a plant or plant part for exhibiting a reduced or absent discolouration as compared to a control plant or plant part, which method comprises

a) providing a plant or plant part that is obtained from a plant population that shows genetic variation, in particular a gene bank;

b) optionally creating a wound surface on the plant or plant part;

c) incubating the plant or plant part or the wound surfaces created thereon 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 assess whether the plant or plant part shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part; and

f) identifying a plant or plant part that shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part as a plant that harbours the reduced wound-induced surface discolouration trait.

In this method 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 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.

Alternatively, variation in discolouration can be detected by using a method for testing a plant or plant part for exhibiting a reduced or absent discolouration as compared to a control plant or plant part, which method comprises:

a) providing a plant or plant part that is obtained from a plant population that shows genetic variation, in particular a gene bank;

b) incubating the plant or plant part 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 assess whether the plant or plant part shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part; and

e) identifying a plant or plant part that shows no discolouration or a discolouration that is reduced as compared to the control plant or plant part as a plant that harbours the reduced discolouration trait.

In the second method 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.

Once suitable plant material harbouring the reduced discolouration trait, in particular the reduced wound-induced surface discolouration trait, has been identified with one of these methods, such plant material is used according to the invention for introducing the trait into commercial crops. Such improved crop plants are also part of this invention.

The present invention is thus based on the use of a specific source of plant material that is tested. Cultured material does not show much variation with respect to discolouration. Unexpectedly it was found that to the contrary germ plasm found in gene banks, such as accessions, do have significant variation in discolouration, in particular wound-surface induced surface discolouration. An accession is one specimen of a species, with a given locality. Accessions are for example stored in gene banks or libraries.

In a preferred embodiment of this invention, the discolouration assays described above are used as an efficient tool to screen for naturally occurring genetic variants in crop species. The invention is in particular useful for testing lettuce germ plasm, for example from the species *Lactuca sativa* as well as related species like *Lactuca seriola*, *Lactuca virosa* etc.

The invention can also be used for crops that are cultivated for leaf or stem parts (endive, witloof), root or stem tubers (potato, sweet potato, celeriac), fruit parts

(eggplant, apple, banana, avocado, peach, pear, apricot, mango), flowers or flower parts (artichoke, gerbera, chrysanthemum) and mushrooms.

Other assays which detect discolouration, in particular wound-induced surface discolouration of lettuce can be used as well in this respect, for example visual assessments of processed lettuce in different maturity stages.

By using such assays, accessions of different *Lactuca* species have been identified which contain significant variation in wound-induced surface discolouration, in particular of the leaves. The individual plants identified by the method described by this invention which have a reduced or absent wound-induced discolouration are used in breeding in order to develop lettuce plants which are improved with respect to their postharvest processing characteristics, especially with respect to wound surface discolouration. Such varietes are especially useful for the convenience market.

In addition, screening of a set of gene bank accessions of eggplant revealed also accessions with reduced discolouration. The use of these accessions in the development of eggplants with reduced discolouration is also part of this invention.

The invention further relates to the use of plants that are identified via the method of the invention as the source of the reduced wound-induced surface discolouration trait. This source can be used as the parent in a conventional cross or as the donor of genetic material that underlies this trait.

Various embodiments relate to use of a lettuce plant as a crop, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic

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material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), wherein the lettuce plant is a progeny of the seed.

Various embodiments relate to use of a lettuce plant as a crop, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained the genetic material that results in the reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586).

Various embodiments relate to use of a lettuce plant as a seed source, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), and wherein the lettuce plant is a progeny of the seed.

Various embodiments relate to use of a lettuce plant as a seed source, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained the genetic material that results in reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession

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651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), into a lettuce plant.

Various embodiments relate to use of a lettuce plant for consumption, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), and wherein the lettuce plant is a progeny of the seed.

Various embodiments relate to use of a lettuce plant for consumption, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained genetic material that results in reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586) into a lettuce plant.

Various embodiments relate to use of a lettuce plant of NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586) in the production of a lettuce plant that shows a reduced brown or pink wound-induced surface discolouration trait as compared to a control plant or a control plant part.

Various embodiments relate to a cell of a lettuce plant, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material,

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which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), and wherein the lettuce plant is a progeny of the seed.

Various embodiments relate to a cell of a lettuce plant, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained genetic material that results in reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586) into a lettuce plant.

Detailed description of the invention

Development of plants, and ultimately plant varieties, with a reduced wound surface discolouration

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through breeding initially requires the identification of genetic variants for this trait. This invention is concerned with this initial identification step and with the further step of introducing the trait into commercial crop plants. Once this first parent plant providing the desired trait has been identified, the development of plant varieties is then a matter of routine for the skilled breeder.

Wound-induced surface discolouration is a specific response of a plant upon cutting or other mechanical disturbances aimed at healing of the wound and defence against pathogenic invasion.

A wound response is a complex biological response of a plant to physical injury which manifests both locally as well as systemically. The local response is mainly aimed at closing the wound surface which is effectuated by the local death of cells at or just behind the cut surface. Lignification or suberisation at the wound surface can be observed in many plant species which leads to an effective new insulation barrier between the plant body and the environment.

In addition to these visible effects, other responses like increased respiration or ethylene production are known to be induced. At the biochemical level studies have shown that wounding can lead to the induction of the phenylpropanoid pathway which is required for *inter alia* the production of polyphenols or lignins.

The key enzyme for this pathway is phenylalanine ammonia lyase (PAL) which is enhanced upon wounding by the induction of gene expression of at least one of the PAL isoforms. This response leads to the formation of polyphenols which are oxidised by polyphenol oxidase (PPO). PPO is residing in the plastids and is released and activated upon wounding. Oxidation of polyphenols leads to the formation of

the highly reactive quinones. These quinones can react with amino acids or proteins which leads to pink, brown or sometimes even black discolouration.

In lettuce, this response can be observed as well. When cut lettuce leaves are stored in a plastic bag in air the cut surface starts to develop a pinkish component at the wound surfaces which gradually turn brown after prolonged storage. Detailed biochemical analysis has shown that this response requires *de novo* synthesis of polyphenols which are subsequently oxidised leading to a cascade of reactions which finally can be observed as a pink or brown colouration at the wound surface.

The postharvest treatments which have been developed and which are currently applied by the lettuce processing industry counteract this particular response. The present invention provides a genetic method to counter this particular response which can be applied to reduce or eliminate postharvest treatments aimed at wound-induced discolouration.

In order to allow development of crops with improved processing characteristics a method is used according to the invention which allows efficient detection of the surface discolouration that is diagnostic for postharvest discolouration of wounded cut crop plants, in particular lettuce.

These methods have been described in co-pending applications PCT/EP2007/000226 and PCT/EP2007/000230.

In a preferred embodiment, leaf discs of lettuce plants are taken and incubated between wetted filter papers. Upon incubation, a pink discolouration will develop which is diagnostic for wound-induced pinking and browning of processed lettuce. Such method therefore allows a very efficient screening of populations of lettuce in which

genetic variability resides in order to determine the presence of genetic variants which have a reduced wound surface discolouration and thereby improved processing quality.

Upon successful identification of such individual plants they can be used for breeding improved lettuce plants and ultimately develop lettuce varieties that are particularly useful for processing.

In order to identify plants with the reduced wound-induced surface discolouration trait natural genetic variation is exploited. Natural genetic variation is the variation in the DNA primary structure which occurs within the germplasm of such species and which is the result of errors during the replication and their maintenance within the DNA of the germinal cells.

The occurrence of such genetic polymorphisms within a population is determined by the fitness of the spontaneous mutation in a given genetic background as well as the selection imposed by the environment.

For lettuce this natural variation is present in the species *Lactuca sativa* but also in other *Lactuca* species such as *Lactuca serriola*, *Lactuca saligna* or *Lactuca virosa*. Plants identified according to the invention can thus also be identified in these species and be used for producing lettuce plants that harbour the reduced wound-induced surface discolouration. The underlying reason is that the natural, interspecific crossing barrier is not absolute and can, if necessary, be overcome by technologies known to the person skilled in the art such as embryo rescue.

Natural variation occurs in the plant material residing at breeding companies or institutes as gene banks in the form of seeds. In order to determine the presence of variants with a reduced wound surface discolouration, the

plant material is raised under normal conditions for example in a greenhouse or in a field. At the young plant stage one can take samples, for example leaf discs, from the individual plants and determine the extent of discolouration using one of the methods described before. This approach for identifying lettuce or other crop plants which are reduced with respect to their wound induced discolouration is by far more efficient as opposed to a method in which the crop is grown to a mature stage, harvested, processed and assessed for processing quality.

In addition, wild relatives of the cultivated lettuce can develop in a completely different manner which makes a comparative analysis less obvious. As it was found that the leaf disc response which manifests as a pink surface discolouration is diagnostic for the post-harvest discolouration, the method of the present invention can identify plants at an early developmental stage on the basis of a biochemical reaction which can be used to improve the post-harvest characteristics of lettuce or other crops.

This approach allows an easier and more reliable comparison of different plant types. It was surprisingly found that within the *Lactuca* genus significant variation for wound induced discolouration can be observed.

The lettuce plants with a reduced level or absence of wound induced discolouration which have been found according to the invention can be used in a regular breeding program to develop varieties tailored to the requirements of the processing industry. In such a breeding program breeders can use regular, well-known breeding methods like QTL-mapping, marker-assisted selection, and back-crossing, to combine the reduced level of wound-induced discolouration with other desired characteristics of cultivated lettuce, like pathogen and pest resistance, absence of bolting and tipburn, etc.

The invention further relates to a method for producing plants with reduced wound-induced surface discolouration, comprising the steps of:

a) crossing a first parent plant with reduced wound-induced surface discolouration with a second parent plant to obtain F1 seed;

b) growing F1 plants from the F1 seeds and self-fertilising the F1 plants to obtain F2-seed;

c) selecting F2 plants for levels of wound-induced surface discolouration that are lower than the levels of at least the second parent plant as plants harbouring the reduced wound-induced surface discolouration trait. These plants and their progeny that retains the reduced wound-induced surface discolouration trait are plants of the invention.

Preferably, the second parent plant is a cultured parent plant having agronomically acceptable characteristics, in particular lettuce.

In a preferred embodiment the first parent is selected from the plants listed in **Table 2**.

The second parent can be any lettuce, but is preferably a cultivated lettuce, such as the iceberg lettuce Silvinas RZ.

The trait that is called herein "reduced wound-induced surface discolouration trait" is present in a plant when the score in the pinking test described in Example 1 is less than 3, preferably less than 2, more preferably 1.

It is now shown that variation is found in naturally occurring populations but it can also be induced by mutagenesis. This creates the possibility to combine different sources of variation to create an unprecedentedly low level of wound-induced discolouration. The combination of different sources of reduced wound-induced variation can be

performed by crossing them, resulting into one or more hybrid seeds. If a hybrid plant from one of those seeds is showing a more reduced wound-induced discolouration, it can be used directly in practice.

In any case, a hybrid plant can be self-fertilised to obtain an F2-population. This F2-population can be used to test for allelism, i.e. both sources provide alleles reduced discolouration on the same locus . In the absence of allelism it is possible to find F2-individuals with a normal, high level, as well as F2-individuals with a more reduced level of wound surface discolouration than both parents. The latter F2-plants carry a combination of alleles for reduced discolouration from both parents and can be used for further breeding.

The process of combining alleles for reduced discolouration can be supported by molecular markers, like AFLPs, SFPs, etc. Additional assays to distinguish different levels of reduced discolouration, like visual assessment of stored, cut leaves of mature plants, can be used to identify the plants with the high reductions in wound surface discolouration resulting from combined alleles from different sources.

In another aspect, it is provided use of a lettuce plant that shows a reduced discolouration, as found in a plant representative seed of which was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) and NCIMB 41569 (*L.sativa* accession 07V.67586), in the production of lettuce plants that show a reduced discolouration and acceptable agronomical characteristics.

The invention will be further illustrated in the Examples that follow and that are given for illustration purposes only. In the Examples reference is made to the following figures:

Figure 1: Leaf disc pinking assay of *Lactuca* accessions. Variability of the response which manifests as different degrees of pink discolouration is observed between the accessions.

Figure 2: Pictures of iceberg lettuce heads of number 07V.67586 with reduced discolouration and cv. 'Silvinas' with normal discolouration.

Figure 3: Browning response of fresh cut eggplant. On the left fruit tissue is shown a few hours after cutting stored at room temperature. On the right fruit tissue is shown which has been incubated for 24 hours at room temperature. The seed browning can clearly be observed.

Figure 4: Cut slices of eggplant and the browning response. On the left two half slices have been incubated at room temperature for 24 hours in 50 mM mes buffer pH = 5.5. On the right the half slices have been incubated under the same condition but with added 10 mM L-cysteine.

Figure 5: Black discolouration of eggplant seeds upon incubation of the seeds in a solution containing 4 mM L-DOPA. Well 1: negative control (buffer only), well 2-6: 4 mM L-DOPA, well 3-6: in addition 0.1, 1.0, 5.0 and 10 mM L-cysteine, respectively.

Figure 6: Screening of genetic material of eggplant for variation in seed-associated PPO activity on the basis of incubation of seeds with the PPO substrate L-DOPA. The wells of columns 2, 4 and 6 contain 4 mM L-DOPA whereas the columns 1, 3 and 5 contain buffer only. Different genetic material in the form of eggplant seeds is assayed in this plate. Each batch of seeds is analysed side by side with and without L-DOPA at the positions A1 and A2, A3 and A4, A5 and A6, B1 and B2, B3 and B4, B5 and B6, C1 and C2, C3 and C4, C5 and C6, D1 and D2, D3 and D4, D5 and D6. The batches at position C5 and C6, D1 and D2, D3 and D4, D5 and D6 show the highest seed-associated PPO activity.

EXAMPLES

EXAMPLE 1

Screening of Lactuca populations for reduced wound surface discolouration

Accessions of lettuce were screened for their potential to display wound induced discolouration. Seeds were germinated in trays containing potting soil in a greenhouse using standard conditions to grow lettuce. Young plants which had developed 3-4 true leaves were sampled using a cork borer. The leaf discs thus obtained were incubated between wetted filter papers in a container at 6°C. After about one week the pink discolouration was scored on a scale from 1 to 5 in which 1 means no detectable pink discolouration and 5 means very clear, maximum level of pink discolouration. The result is shown in **Table 1**. This table shows the internal reference and their respective score. Accessions that were confirmed to have a reduced pink discolouration are identified below.

An example of the observed differences in the pinking reaction of different accessions using the pinking leaf disc assay is shown in **Figure 1**.

Surprisingly, it was found that several accessions showed reduced discolouration. This was unexpected, because this trait is considered a disadvantage under natural and grower's conditions as the process resulting in discolouration is considered to protect the plant against adverse biotic and abiotic stress factors entering through a wounded spot.

The most interesting accessions from this test were resown and retested according to the same protocol as mentioned above. Reduced pinking was confirmed for number 651968 (L.virosa; plot nr. 366, NCIMB 41489). Additional accessions were tested according to the same protocol as

mentioned above and retested for confirmation. Reduced pinking was found and confirmed for number 650147 (L.saligna; NCIMB 41485). The accessions that were found to show reduced wound-induced surface discolouration are listed in **Table 2.**

Well-known accessions such as 'Iceberg' and 'Salinas' were not showing any signs of reduced pinking in this test.

Table 2

Overview of accessions with reduced discolouration of wound surfaces.

number	species	type	accession number	deposit date
651968	*L.virosa*		NCIMB 41489	July 31, 2007
650147	*L.saligna*		NCIMB 41485	July 31, 2007
07V.67586	*L.sativa*	crisphead	NCIMB 41569	July 2, 2008

Seeds of the numbers 651968, 650147 and 07V.67586 were deposited with NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, Scotland, AB21 9YA, UK.

Table 1

Pinking score of different *Lactuca* accessions. Each field number refers to an individual accession. Two plants per accession were sampled. The score indicates the level of pinking: 1 = no pinking to 5 = strong pinking. In case the two plants differed in pinking two scores are indicated. In case one score is indicated the two plants reacted identical in the test.

Veld nr.	Score	Veld nr.	Score	Veld nr.	Score	Veld nr.	Score
1	5	195	5 en 4	257	4	319	4
2	3	196	5	258	4	320	5
3	3	197	5	259	5	321	5 en 2
4	3	198	5	260	5	322	5 en 2
5	5	199	5	261	4	323	2 en 1
6	5	200	5	262	4	324	4 en 3
7	5	201	5	263	4	325	5
8	4	202	5	264	4	326	5
9	4	203	5	265	4	327	4
10	4	204	5	266	4	328	2
11	3	205	5	267	5	329	2 en 3
12	4	206	5	268	4	330	1
13	5	207	5	269	4	331	2
14	5	208	5	270	4	332	3
15	5	209	5	271	5	333	2 en 3 en 5
16	5	210	5	272	5	334	5
17	3	211	5	273	5	335	5
18	5	212	5	274	5	336	5
19	5	213	5	275	5	337	3
20	5	214	5	276	1 en 3	338	5
21	2	215	5	277	2 en 5	339	4
154	5	216	5	278	1 en 4	340	5
155	5	217	5	279	5	341	3
156	5	218	5	280	5	342	2 en 1
157	5	219	plant nog te klein	281	5	343	4
158	5	220	4	282	5	344	5
159	4	221	4	283	5	345	5
160	5	222	4	284	3	346	5
161	5	223	4	285	5	347	4
162	5	224	3 en 4	286	5	348	5
163	5	225	4	287	3 en 5	349	5
164	4	226	4	288	5	350	5
165	5	227	4	289	5	351	5
166	5	228	5	290	5	352	5
167	5	229	5	291	3 en 5	353	4
168	5	230	5	292	5	354	5
169	5	231	4	293	5	355	4
170	5	232	4	294	5	356	5
171	5	233	4	295	5	357	4
172	5	234	4	296	5	358	4
173	5	235	4	297	5 en 4	359	3
174	5	236	2 en 4	298	2 en 4	360	4
175	5	237	2	299	4	361	4
176	4	238	4	300	2 en 3	362	4
177	2	239	4	301	4	363	3
178	5	240	4	302	4	364	3
179	2	241	4	303	4	365	3
180	5	242	4	304	5	366	2
181	5	243	4	305	5	367	2
182	5	244	5	306	5	368	3
183	5	245	5	307	5	369	2
184	3	246	5	308	5	370	4
185	5	247	3 en 4	309	5	371	4
186	5	248	4	310	5	372	3
187	5	249	5	311	5	373	3 en 2
188	5	250	4	312	5	374	3
189	5	251	4	313	2 en 5	375	4
190	5	252	4	314	3 en 5	376	5
191	5	253	4	315	5	377	5
192	5	254	4	316	5	378	5
193	5	255	4	317	5 en 3	379	5
194	5	256	4	318	5	380	5

EXAMPLE 2

Field screening

A field screening was performed by visual assessment of cut crisphead lettuce heads. In one accession reduced discolouration of wound-surfaces was observed (**Figure 2**). This accession has number 07V.67586. It was re-tested by visual assessment in a following field trial and was confirmed to have reduced discolouration. This visual assessment was done one day after cutting. Surprisingly a follow-up test according to the protocol mentioned in Example 1 did not show reduced pinking. This could be an indication for a different genetic cause of reduced discolouration in number 07V.67586 than in the other accessions described in Example 1.

EXAMPLE 3

Reduced discolouration in eggplant

Upon cutting and packaging of eggplant fruits a rapid browning reaction can be observed. The wound surface of the cut fruit tissue rapidly turns brown which reduces the quality of the product. More importantly the seeds embedded within the fruit flesh show a strong brown discolouration which stands out against the background as shown in **Figure 3**.

For industrial use of fresh cut eggplant or the use of eggplant as fresh produce, seed browning needs to be prevented. In order to determine the biochemical nature of the brown discolouration of cut eggplant fruits, the browning reaction was observed both with and without L-cysteine which is a strong inhibitor of polyphenol oxidase (PPO). The result of this analysis is shown in **Figure 4**.

The result shown in **Figure 4** demonstrates that the browning reaction can be inhibited by the exogenously

supplied PPO inhibitor L-cysteine. Therefore, the browning response is clearly PPO-mediated.

In order to prevent the brown discolouration in eggplant caused by the seeds using a genetic approach, genetic material was screened for variation in seed-associated PPO activity. For that purpose the method was used that relies on the conversion of a substrate, such as L-DOPA, into a coloured pigment, such as melanin.

Upon incubation of eggplant seeds in a solution containing dihydroxyphenylalanine (L-DOPA) as a PPO substrate, melanin formation clearly visible as a black discolouration occurs. This black discolouration is PPO-mediated because it can be completely inhibited by L-cysteine which is shown in **Figure 5**.

The L-DOPA mediated seed discolouration of eggplant is used to identify genetic material which has reduced seed-associated PPO activity. This screening resulted in the identification of genetic material which shows a significant reduction of the seed-associated PPO activity. The result of such screening is illustrated in **Figure 6**.

The eggplant material which has been taken through this screening method and which has shown a strong reduction in seed-associated PPO activity is summarised in **Table 3**. In this screening some breeding lines show a considerable reduction in seed-associated PPO activity (13, 14, 42, 49), whereas others show strong browning (7, 27, 34, 47).

Table 3

Number	Breeding line/code	Reduced PPO activity
13	AB5480	+
14	AB5701	+
19	05N.1130-1	+

22	SNL.109	+
24	06N.1268-1	+
25	SNL.111	+
42	AB5028	+
49	AB5103	+
7	AB5261	-
27	06N.244-2	-
34	AB5007	-
47	AB5101	-

By making crosses between two lines which show this reduction in seed-associated PPO activity, hybrids are obtained that are more suited for both industrial as fresh market use. Furthermore, wild accessions have been found that are either closely related to eggplant (19) or more distantly related (22, 24, 25) and which show a very strong reduction in seed-associated PPO activity. These accessions are used as a start of a backcross program which results in breeding lines and hybrids that are even more suitable for both industrial as fresh market purpose. In the backcross process, the method of screening mentioned above is used to assist in selection for the strongest reduction in seed-associated PPO activity.

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

1. Use of a lettuce plant as a crop, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), wherein the lettuce plant is a progeny of the seed.

2. Use of a lettuce plant as a crop, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained the genetic material that results in the reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586).

3. Use of a lettuce plant as a seed source, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna*

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accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), and wherein the lettuce plant is a progeny of the seed.

4. Use of a lettuce plant as a seed source, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained the genetic material that results in reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), into a lettuce plant.

5. Use of a lettuce plant for consumption, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), and wherein the lettuce plant is a progeny of the seed.

6. Use of a lettuce plant for consumption, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained genetic material that results in reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB

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41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586) into a lettuce plant.

7. Use of a lettuce plant of NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586) in the production of a lettuce plant that shows a reduced brown or pink wound-induced surface discolouration trait as compared to a control plant or a control plant part.

8. A cell of a lettuce plant, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the reduced brown or pink wound-induced surface discolouration is caused by genetic material, which genetic material is as found in seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586), and wherein the lettuce plant is a progeny of the seed.

9. A cell of a lettuce plant, the lettuce plant showing a reduced brown or pink wound-induced surface discolouration, wherein the lettuce plant has obtained genetic material that results in reduced brown or pink wound-induced surface discolouration by transferring the genetic material from seed of which a representative sample was deposited under accession number NCIMB 41489 (*L.virosa* accession 651968), NCIMB 41485 (*L.saligna* accession 650147) or NCIMB 41569 (*L.sativa* accession 07V.67586) into a lettuce plant.

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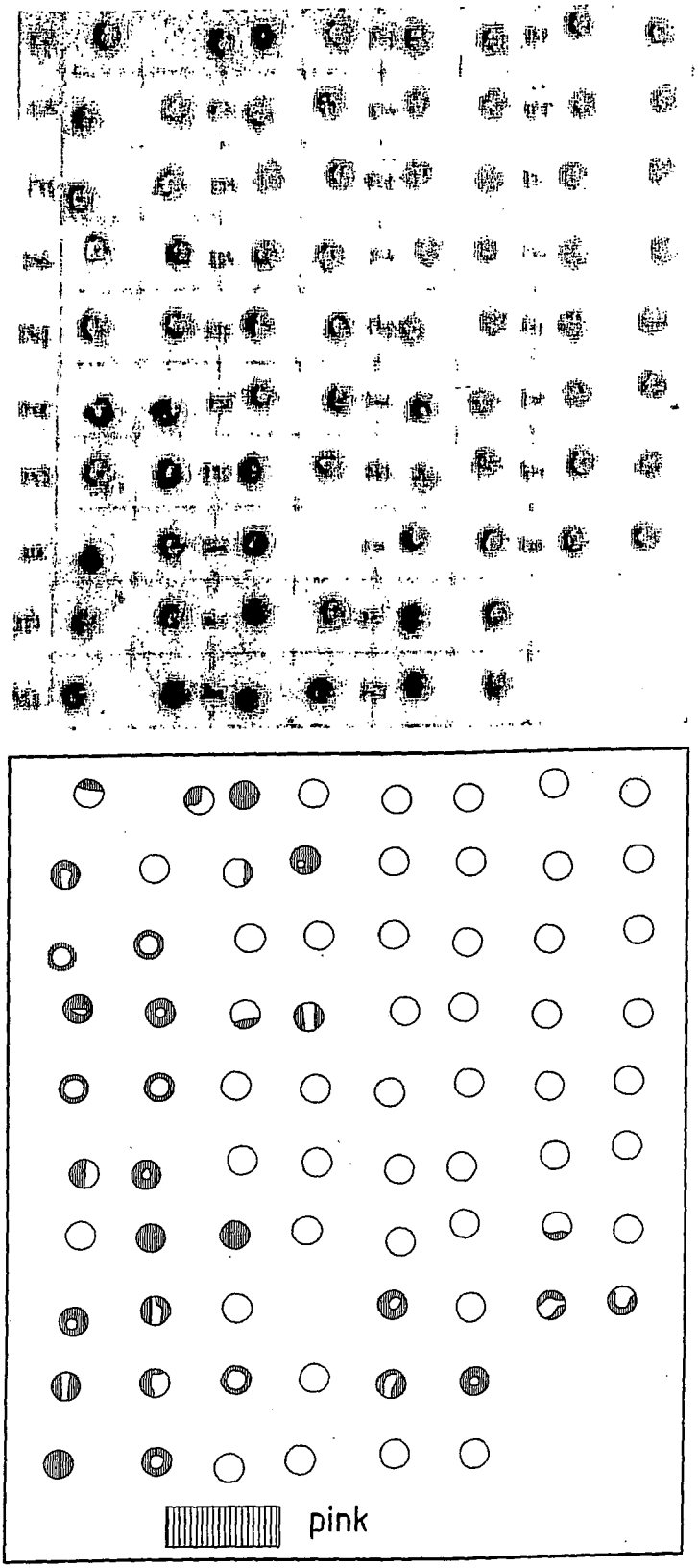
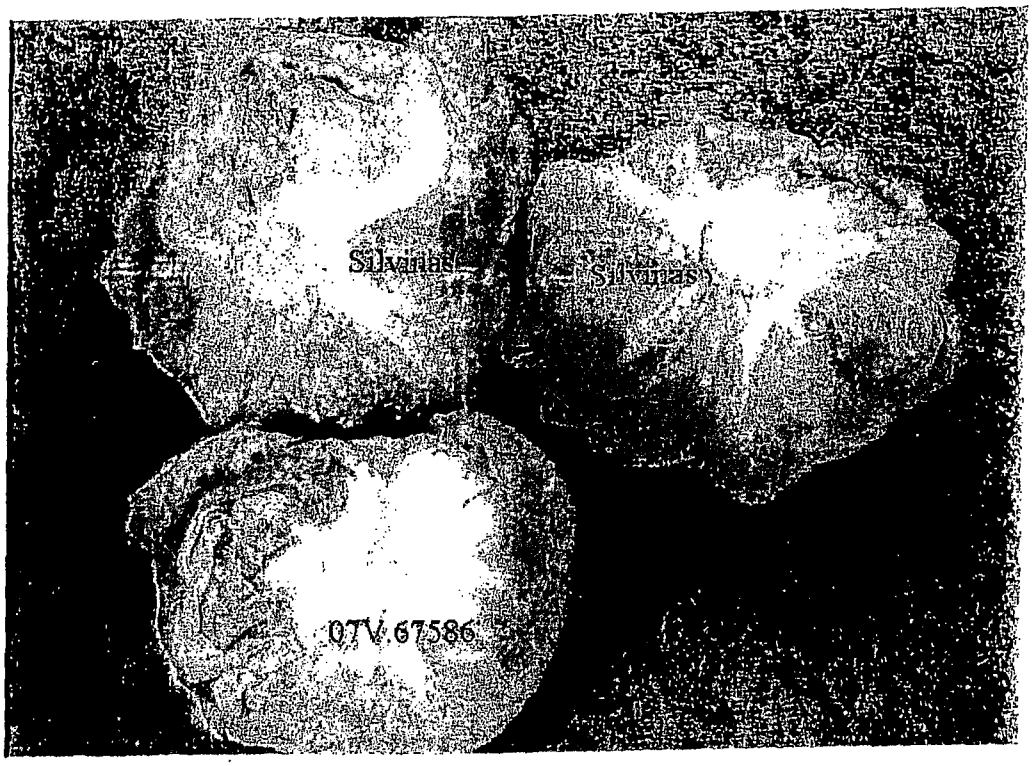


FIG. 1



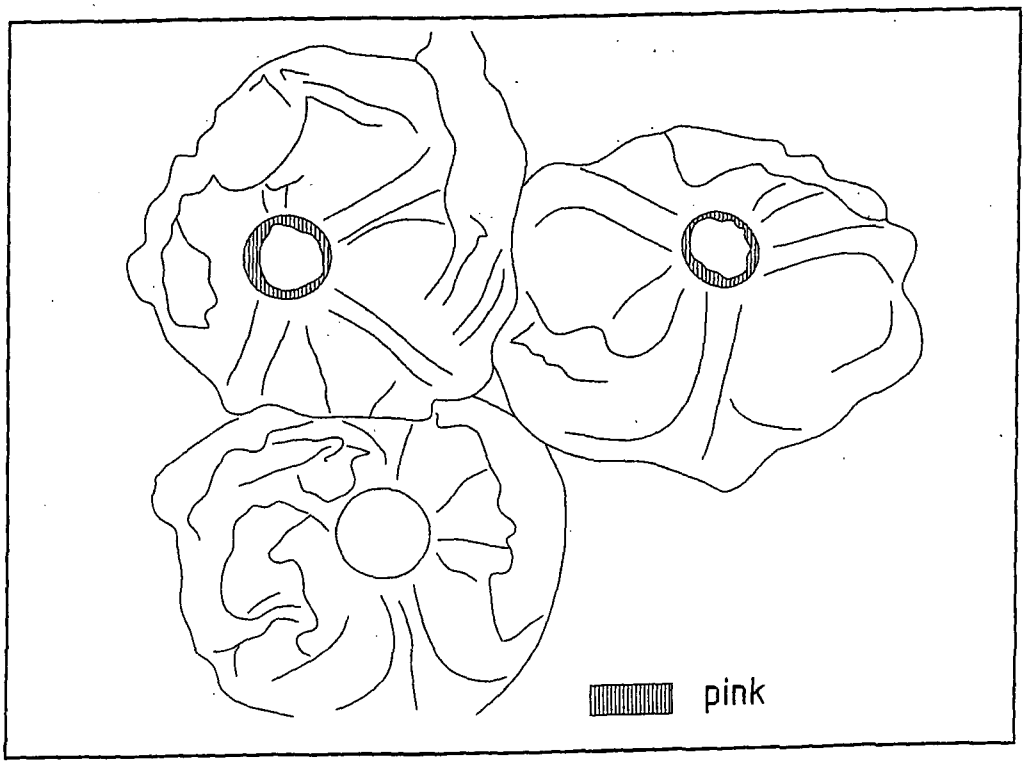


FIG. 2

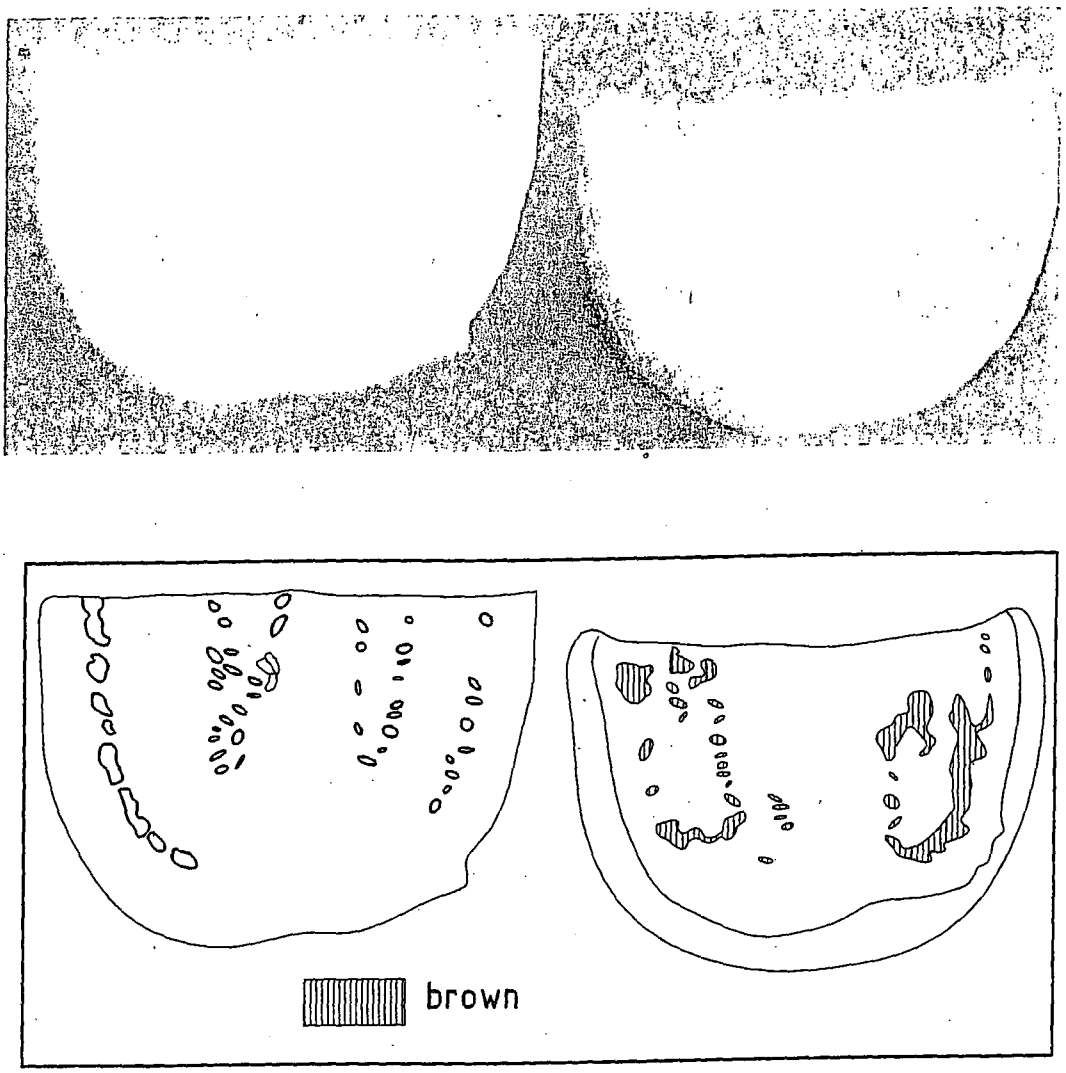


FIG. 3

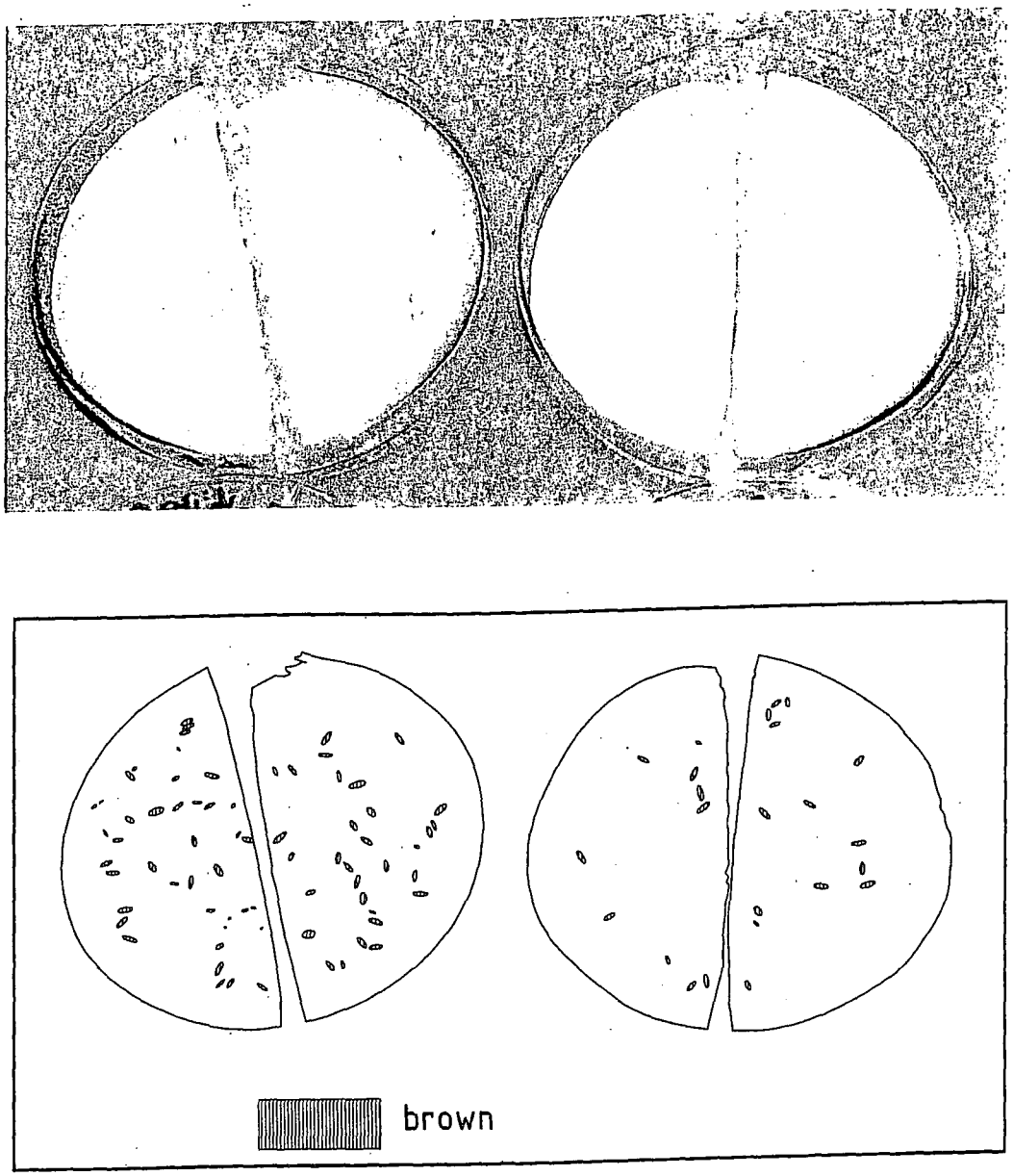


FIG. 4

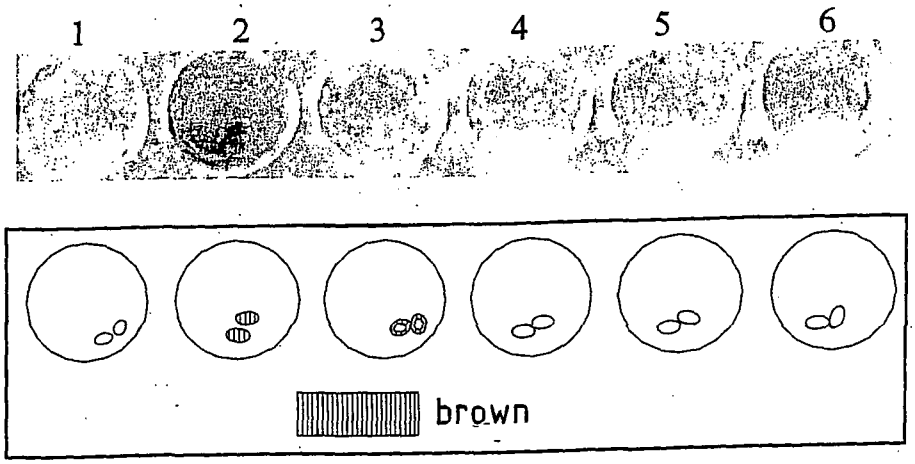


FIG. 5

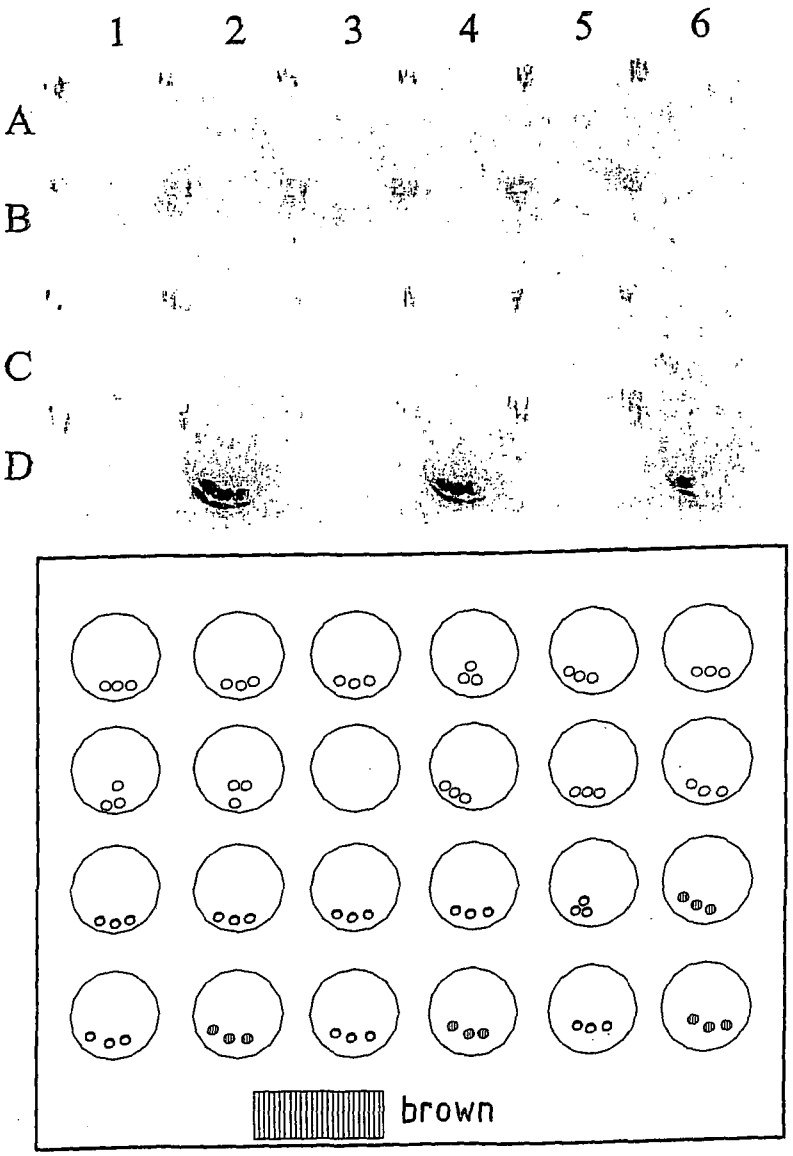
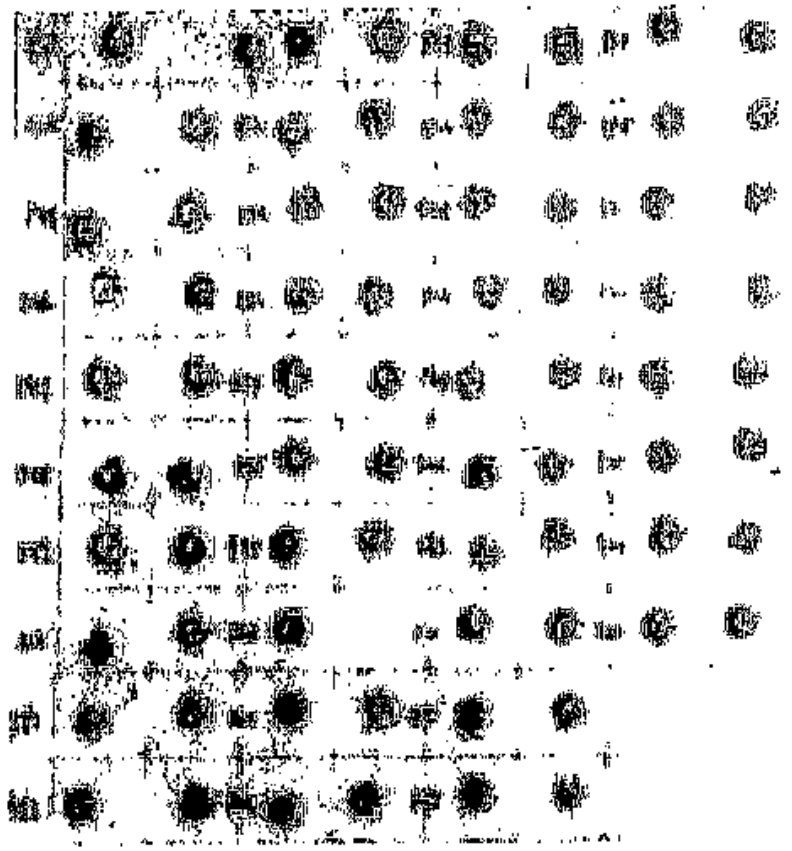
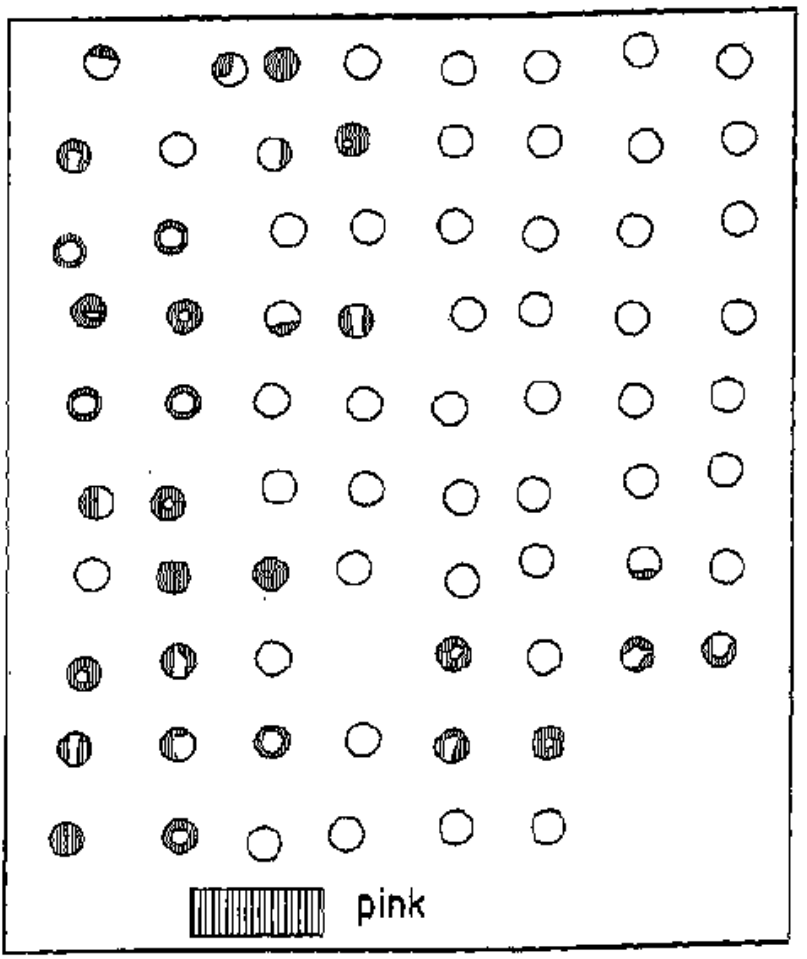


FIG. 6




pink