This invention relates to a process for the production of substances of vegetable origin by "in vitro" culture.

Numerous substances of vegetable origin have a certain economic value for such industries as the pharmaceutical industry (active principles of medicaments), the cosmetics industry (perfume) and the food industry (flavourings and additives). The substances in question are generally produced by extraction from collected or cultivated plants. However, this method has many disadvantages, particularly in regard to restocking of the vegetable starting material (periodicity, stability, quality, etc.).

Various substitute processes have been investigated with a view to obviating these disadvantages. They include the "in vitro" process for culturing undifferentiated tissues or cells of productive species. Reference is made in this regard to Japanese patent JP 57-39778, which relates to the culture of calluses in an agitated liquid medium containing cellulose to extract the active principles therefrom with paraffin oil and/or fats, and to the Article by Bisson et al (Journal of Medicinal Plant Research, 1983, 47, 164) which relates to the extraction of essential oils from a culture of undifferentiated cells of camomile by addition to the nutrient medium of droplets of a fatty phase in which the oils are to accumulate. However, because they use undifferentiated cells, these techniques are limited to the preparation of well-defined products.

The object of the present invention is to combine the advantages of the differentiated system with those of "in vitro" culture.

To this end, the process according to the invention is characterized in that

- a vegetable organ is implanted in a culture medium and
- is kept under anoxia or hypoxia by immersion beneath a layer of oil.

The substances produced are preferably extracted from the vegetable organ, from the culture medium and/or from the oil. Conventional extraction techniques may be used for this purpose. The main advantage of the process lies in the "in vitro" culture of vegetable organs producing the desired substances and in their sustenance and their production of these substances over long periods.

The oil used to maintain hypoxia may enable possible losses of volatile substances to be avoided and the substances in question to be extracted.

One advantage of this invention is that it enables the substances to be produced in a constant and reproducible manner. Another advantage of the invention is that it enables the production phase of the substances by vegetable organs to be sustained for much longer periods than the normal duration of "in situ" production by plants.
Another advantage of the invention is that it enables the vegetable organ to retain its specific qualitative characteristics, which is rarely the case with undifferentiated cell cultures where secondary substances may be produced.

In this way, the vegetable organs not only do not become cankered and survive for several months, they also continue to accumulate in their tissues, the culture medium and the hypoxia oil such substances as essential oils identical or similar in nature to those produced by traditional methods of extraction from plants.

The process according to the invention is concerned with the sustenance of vegetable organs and not with the growth and/or proliferation of tissues, although this may occur to a reduced extent in certain cases.

Suitable criteria for evaluating the survival of the vegetable organ include

- as appearance criteria:
  - the development of pigmentation in the implant
  - the appearance of chlorosis or browning (oxida-

tion),

- as morphological criteria:
  - blooming
  - the appearance of calluses and/or roots.

These criteria are evaluated in different ways, namely:

- visually
- by biochemical tests (coloration test, etc.), which enable the viability of the organ to be assessed.

The vegetable organs cultured may be of any type and may be cultivated in part or as a whole. They may be, for example, fragments of roots, leaves or stems or even flower buds or opened flowers.

The plants or organs may be of any origin, i.e. are

- either obtained by harvesting of cultivated or collected plants, in which case the process enables utilization of traditional vegetable production to be improved by extension of the biosynthesis and production phase of the desired substances,
- or formed from cell cultures, in which case the process provides for complete independence from traditional culture.

The plants or vegetable organs may be decontaminated before their implantation in the culture medium to eliminate troublesome microorganisms. The plants or organs may be decontaminated by immersion for a few minutes in a decontaminating solution, such as Javel water, mercury dichloride, calcium or sodium hypochlorite or any other typical decontaminant or by any other chemical or physical means (X rays, γ rays, etc.).

The culture medium used should provide the vegetable organ with the nutrients necessary for its survival in the production phase. This nutrient medium may be in semi-solid form (aqueous gelose) or in the form of an optionally stirred liquid to simplify culture of the vegetable organs. In the latter case, culturing may merely amount to immer-

sion of the decontaminated organs in the nutrient medium followed by covering with a film of oil to avoid any changes in the surface.

The culture medium may be renewed so that the organ is always supplied with the elements necessary for its survival and to remove any diffused compounds (antagonistic and/or desired) therefrom. Renewal of the culture medium may be continuous or semi-continuous.

Similarly, the culture medium may be modified by addition of growth regulators and/or by addition of elements capable of promoting production of the desired metabolites (addition of precursors).

The culture medium is preferably a medium of the type typically used in the "in vitro" culture of tissues, such as a Gamborg medium, Heller medium or even Murashige and Skoog medium, of which the compositions are shown in Table I below. The preferred culture medium according to the invention is Gamborg medium.

The oil used to cover the vegetable organ may perform two functions, namely:

- it acts first and foremost as a preservative, ensuring the maintenance of hypoxia and
- it may act as a trapping/extracting agent for the desired lipophilic substances.

The choice of the oil is dependent on certain criteria, such as:

- its ability to cause hypoxia, i.e. to transfer no external oxygen or very little external oxygen to the vegetable organ and its culture medium,
- its dissolving power on the desired substances,
- its absence of toxicity with respect to the vegetable organs.

Since it is preferred to renew the oil periodically, because it can become saturated by extraction of the desired substances, a liquid hypoxia medium is easier to

handle than a solid medium, although a solid medium may be used. The oil may be renewed continuously or semi-continuously. Suitable oils include oils of organic or vegetable origin, synthetic oils and any other oil capable of acting as a preservative. The preferred oil according to the invention is a paraffin oil. The oil may be degassed and sterilized before use. Degassing may be carried out by placing the oil under vacuum, the gases present therein escaping and optionally being replaced by nitrogen.

To avoid the degradation of chlorophyll, which could adversely affect survival of the vegetable organ, low lighting of the order of 0-400 lux may be used in the application of the process according to the invention. The culture temperature may be in the range from 18°C to 35°C and is preferably of the order of 25°C.

The process according to the invention for the production of substances of a vegetable origin may be carried out discontinuously, semi-continuously or continuously.

The invention is illustrated in more detail in the following Examples. Examples A, B and C relate more particularly to sustenance and to the criteria used for its evaluation. The Examples are preceded by the following Table which shows the composition of the culture medium used.

Table I: Composition of the media used (pH = 5.8)

	Murashige and Skoog medium mg/l	Gamborg medium mg/l	Heller medium mg/l
Sucrose	20000	20000	20000
Agar (media treated with gelose)	8000	8000	8000
Macro elements			
$NH_4 NO_3$	1650	-	-
$(NH_4)_2SO_4$	-	134	-
$CaCl_2 \cdot H_2O$	440	150	75
$MgSO_4 \cdot 7H_2O$	370	250	250
KCl	-	-	750
KNO_3	1900	2500	-
KH_2PO_4	170	-	-
$NaNO_3$	-	-	600
$NaH_2PO_4 \cdot H_2O$	-	-	125
Micro elements			
$AlCl_3 \cdot 6H_2O$	-	-	0.05
$CoCl_2 \cdot 6H_2O$	0.025	0.025	-
$CuSO_4 \cdot 5H_2O$	0.025	0.025	-
$FeCl_3 \cdot 6H_2O$	-	-	1.0
$FeSO_4 \cdot 7h_2O$	27.8	27.8	27.8
Na_2 -EDTA	37.3	37.3	37.3
$MnSO_4 \cdot 4H_2O$	22.3	10.0	0.1
$NiCl_2 \cdot 6H_2O$	-	-	0.03
KI	0.83	0.75	0.01
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	-
$ZnSO_4 \cdot 7H_2O$	8.6	2.0	1.0
H_3BO_3	6.2	3.0	1.0
Vitamins			
Calcium pantothenate	-	-	1
Meso-inositol	100	100	100
Nicotinic acid	10.5	1	1
Pyridoxine (vit. B_6)	0.5	1	1
Thiamin (vit. B_1)	0.1	10	1
Biotin	-	-	0.1

Example A

Fragments of fennel stems are decontaminated by immersion for a few minutes in a solution of calcium hypochlorite and thorough rinsing in sterile distilled water. These fragments are then implanted in a Gamborg medium solidifed by addition of agar agar. They are then placed under hypoxia by addition of sterilized paraffin oil and left standing at 25°C in low light (200 lux). The evolution of the pigmentation of the fennel stems placed under hypoxia by comparison with similar stems placed in the same nutrient medium, but without the added oil, is observed over a period of 18 weeks.

After 6 weeks, all the stems under hypoxia are still green without any sign of chlorosis whereas the comparison stems all show incipient chlorosis. 30% of the comparison stems are affected by oxidation or total chlorosis after 8 weeks whereas only 10% of the stems under hypoxia show incipient chlorosis after the same period and only 20% after 12 weeks. At 16 weeks, 90% of the comparison stems are completely oxidized against 40% for the stems under hypoxia, the rest still remaining green. After 18 weeks, 100% of the comparison stems are completely oxidized whereas only 50% of the stems under hypoxia are completely oxidized, the remaining 50% showing only incipient chlorosis. Finally, after 6 weeks, none of the comparison stems is completely green whereas 50% of the stems under hypoxia are still completely green after 16 weeks.

Example B

Flower buds of tuberose are collected at three different stages of development, namely:

stage I: corolla 1-1.5 cm long - green petals

stage II: corolla 2 cm long - beginning of whitening of the petals

stage III: corolla 3 cm long - white petals

They are decontaminated and implanted in a Gamborg medium treated with gelose. The buds are then placed under hypoxia by addition of paraffin oil. No oil is added to the comparison buds. The whole is left under low light (200 lux) at 25°C.

The evolution of the oxidation of these flower buds as a function of time is recorded arbitrarily on a scale of 0 to 7 in which

0 = absence of oxidation patches

1 = beginning of oxidation at the ends of the petals on at most 10% of the petals

2 = beginning of oxidation at the ends of the petals on more than 10% of the petals

3 = oxidation of half the surface area of the petals on at most 10% of the petals

4 = oxidation of half the surface area of the petals on more than 10% of the petals

5 = oxidation of half the surface area of the petals on more than 50% of the petals

6 = significant oxidation of the flower (80%)

7 = complete oxidation of the flower.

It should be noted that the addition of antioxidants to the paraffin oil does not bring about any significant reduction in oxidation.

The use of degassed oil does not produce any significant difference in relation to non-degassed oil. By contrast, there is a clear difference between the buds placed under hypoxia and the comparison buds.

The following Tables show the evolution of the degree of oxidation as a function of time:

For the buds at stage I:

Time (days)	2	5	7	10	13	17	18	24	28
Comparison	0	0	1	1	5	5	6	7	7
Oil	0	0	0	1	1	1	2	2	3
Degassed oil	0	0	0	1	1	1	2	2	3

It can be seen that the comparison buds are completely oxidized after 24 days whereas the buds under hypoxia survive for more than 6 weeks.

For the buds at stage II:

Time (days)	2	5	7	10	13	17	18	24	28
Comparison	0	0	2	2	5	7	7	7	7
Oil	0	0	0	1	1	1	1	2	2
Degassed oil	0	0	0	1	1	1	1	2	2

The comparison buds are oxidized after 17 days whereas the buds under hypoxia are only slightly oxidized after 4 weeks.

For the buds at stage III:

Time (days)	2	5	7	10	13	17	18	24	28
Comparison	2	5	7	7	7	7	7	7	7
Oil	0	0	1	1	2	3	3	4	4
Degassed oil	0	1	1	2	3	4	4	5	5

The comparison buds are oxidized after 7 days whereas the buds under oil can be kept for up to 28 days before being completely oxidized.

Example C

Various vegetable organs were decontaminated, implanted in a Gamborg medium treated with gelose, then placed under hypoxia beneath a layer of paraffin oil and left at 25°C in low light (200 lux). The vegetables include aromatic plants (I), perfume plants (II) and plants which do not produce an aroma, but do produce non-volatile substances of pharamcological or cosmetic value (III).

The results of these tests are shown in the following Table:

	Species	Organs	Possible survival under hypoxia
I	• Ocimum basilicum	leaves	10-12 weeks
	• Verbena triphylia	leaves	3-4 weeks
II	• Pelargonium capitatum	leaves	8-10 weeks
	• Pogostemon patchouli	leaves	8-10 weeks
III	• Ferlua galbanum	roots	10-12 weeks appearance of calluses
	• Gingko biloba	leaves	14-16 weeks

Irrespective of the type of plant or organ implanted, survival under hypoxia is possible for at least one month and may be maintained for as long as four months.

Example 1

Mint leaves which have been decontaminated and then implanted in a Gamborg medium are placed under hypoxia beneath a layer of paraffin oil at 25°C. The production of aromatic compounds during the survival phase is followed over a period of 5 weeks. The mint leaves are divided into three groups and subjected to different experimental conditions:

group A: strong light (2000 lux)

group B: low light (200 lux)

group C: strong light (2000 lux) and addition of terpene precursor.

The presence in the oil of a compound characteristic of the essential oil, namely carvone, is particularly followed. The oil is regularly renewed over a period of 35 days in the three groups and a quantitative analysis is performed by densitometry through thin-layer chromatography to determine the quantity of carvone present in the oil collected. The results are confirmed by analysis by gas-phase chromatography. The following Table shows the quantity of carbon which has collected in the oil for the three groups over a period of 5 weeks. The quantity of carvone is expressed in micrograms per gram fresh implanted leaf material.

Time (days)	1	2	3	4	7	14	21	28	35
Group A	20	60	10	240	10	170	120	240	110
Group B	110	60	10	20	20	20	20	140	110
Group C	620	60	80	10	170	170	260	340	170

A peak is observed in the production of carvone at the beginning of kinetics, followed by a fall in production and

then by a resumption with a new accumulation peak at 28 days.

The following Table shows the increase in the accumulation of carvone over the 35 day period (in µg/g fresh material).

	I	II	III	IV
Group A	690	980	635	925
Group B	690	510	600	520
Group C	690	1880	820	2010

I: initial carvone content in the implanted leaf
II: carvone accumulated in the oil over 35 days
III: final carvone content of the residual tissues
IV: increase in carvone over 35 days (II + III - I).

These figures clearly illustrate the resumption in the synthesis of carvone over a period of time rather than a simple solid/liquid extraction phenomenon; the total produced may reach three times the quantity present in the initial implant.

Example 2

Fennel stems according to Example A are cultivated in vitro under paraffin oil in a Gamborg medium treated with gelose. The synthesis of p-allyl anisole, a compound characteristic of the essential oil, during the survival phase is followed over a period of 2 months.

The fennel stems are divided into three groups which are subjected to different experimental conditions:

Group A: basic Gamborg medium
sucrose: 20 g l^{-1}
lighting 200 lux

Group B: basic Gamborg medium + growth regulator

sucrose: 20 g l^{-1}
lighting: 200 lux

Group C: 1/10 basic Gamborg medium
sucrose: 10 g l^{-1}
lighting: 200 lux

The paraffin oil is regularly renewed after 1, 3, 5 and 7 weeks and then analyzed quantitatively and qualitatively by gas-phase chromatography. The presence of p-allyl anisole in the oil and the appearance of numerous other aromatic compounds are noted.

The following Table shows the quantity of p-allyl anisole which has accumulated in the oil (in μg/g of fresh implanted leaf material) as a function of time:

	Time (days) 7	21	35	49	I	II	III	IV
Group A	222	42	122	traces	333	386	35	88
Group B	400	48	42	traces	333	490	30	187
Group C	291	94	52	136	333	573	106	346

I: initial p-allyl anisole content of the implanted stem
II: p-allyl anisole accumulated in the oil
III: final p-allyl anisole content of the residual tissues
IV: increase in p-allyl anisole over 7 weeks (II + III - I)

The total production can reach twice that of the implant (group C).

CLAIMS

1. A process for the production of substances of vegetable origin by "in vitro" culture, characterized in that
   - a vegetable organ is implanted in a culture medium and
   - is kept under hypoxia by immersion beneath a layer of oil.

2. A process as claimed in claim 1, characterized in that the substances of vegetable origin produced are extracted from the vegetable organ, the culture medium and/or the oil.

3. A process as claimed in claim 1, characterized in that the culture medium and/or the oil are renewed continuously or semi-continuously.

4. A process as claimed in claim 1, characterized in that the oil is an oil of organic origin, an oil of vegetable origin or a synthesis oil and in that it is sterilized and/or degassed.

5. A process as claimed in claim 4, characterized in that the oil is a paraffin oil.

6. A process as claimed in claim 1, characterized in that the vegetable organ is all or part of a root, leaf, flower or flower bud.

7. A process as claimed in claim 1, characterized in that the vegetable organ is decontaminated before implantation.

8. A process as claimed in claim 1, characterized in that precursors and/or growth regulators are added to the culture medium.

9. A process as claimed in claim 1, characterized in that the organ is kept under hypoxia in low light of 0 to 400 lux.

10. A process as claimed in claim 1, characterized in that the organ is kept under hypoxia at a temperature in the range from 18 to 35°C.

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