In vitro mutagenic studies of *Portulaca* spp.

Synopsis

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INTRODUCTION:

Plant Tissue culture is a technique which is conventionally used for conservation, propagation of plants, for the production of secondary metabolites which are potential source of drugs, flavor and fragrances, food additives, dyes and pigments.Explants, such as leaves, stems, roots, and meristems can be used for multiplication and extraction of secondary metabolites (Hussain et al 2012) such as vindoline, catharanthine, vincristine in *Catharanthus roseus* (Ataei- Azimi et al 2008), *β*-sitosterol and caffeic acid in *Sericostoma pauciflorum*(Jain et al 2012), iridoids, phenolics, flavonoids and tannins in *Aloe arborescens* (Amoo 2012).

The quality of secondary metabolites in plants can be improved by mutation with the use of physical and chemical mutagens(Gottschalk and Wolff 1983, Kuckuck et al 1991)such as oil content in *Brassica napus* (Rahimi and Bahrani 2011) and tannins in *Psophocarpus tragonolobus* (Klu et al 1997). Gavidia and Perez-Bermudez (1999) have opined that limited work has been done on the application of mutation technique to induce new varieties of medicinal plants overproducing the desired secondary metabolite. Secondary metabolite under the influence of mutagens have been manipulated under in vitro conditions such as Artemisin in *Artemisia* from ^{12}C ion beam (Inthima et al 2014), Allylisothiocynate in *Wasabia japonica* from X rays and gamma rays (Hung and Johnson 2008), cardenolide content in *Digitalis obscura* from gamma rays (Gavidia and Perez- Bermudez 1999),and BacosideA in *Bacopa .monnieri* from EMS (Naik et al 2012). Thus in vitro mutagenesis has a potential to increase genetic variability and large number of mutant population in vitro can be developed according to the regeneration system (Maluszynski 2001).

The present study deals with two medicinally and nutritionally important plants *Portulaca oleracea* and *Portulaca grandiflora* belonging to the family Portulacaceae.

P oleracea is a rich source of omega 3 fatty acid with a number of health benefits such as decreasing the incidence of cardio vascular diseases and cancer (Cave 1991 and Simopolous et al 1992). It is also essential for visual functions, neural development (Dyall et al 2008) and contains high level of vitamin E, C and betacarotenes (Simopolous 1991).

Portulaca grandiflora is a common ornamental herb growing extensively during summers. The plant is rich in betalains which is present mostly in the stem. This compound is antifungal (Kimler 1995), antioxidant (Escribano et al. 1998) and is known to have positive health effects on humans (Tesoriere et al. 2004). Its wide range of pH stability also makes it an important natural color in the juice industry.

The present work investigates regeneration from leaf explants, secondary metabolite contents, and the effect of mutagens on secondary metabolite content in both the species.

OBJECTIVES:

P oleracea

(1) Regeneration from in vivo and in vitro leaf explants

(2) Fatty acid analysis from in vivo plants and in vitro shoots culture

(3)Effect of mutagens on fatty acid content in shoot cultures.

P grandiflora

(1) Regeneration from in vivo and in vitro leaf explants

(2) Betalain analysis from in vivo plants

(3) Effect of mutagens on betalain.

MATERIALS AND METHODS

The present investigation was conducted on *P oleracea* and *P grandiflora*. The explants for establishing cultures were obtained from plants growing in the Botanical Garden of Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara. The study is divided into three parts for both the plants:

(A) Regeneration Studies

(B) Secondary Metabolite Studies

(C) Mutation Studies

(A) REGENERATION STUDIES

Regeneration studies were carried out for both the species of *Portulaca* ie *P oleracea* and *P grandiflora* on Murashige and Skoogs medium (1962) which was supplemented with 3% sucrose, PGR's and 8% agar. The pH of the medium was set to 5.8 and autoclaved at 121^0C for 20 minutes. The explants was washed in running water for 1 hour and then with a mild detergent. Surface sterilization of the explants was done in $HgCl_2$ for 3 minutes. This was followed by inoculation under laminar hood flow. The cultures were kept in culture rooms fitted with white fluorescent lights.

(I) Regeneration from leaf explants

1) Establishment of shoot cultures in *P oleracea* and *P grandiflora*

Regeneration from the leaf explants of both the species of *Portulaca* was achieved from

(i) In vivo leaf explants

(ii) In vitro leaf explants

(i) In vivo leaf explants (*P oleracea* and *P grandiflora*)

The leaf explants was placed abxially on the sterilized M.S medium supplemented with the following PGR's.

(a) Basal Media (Control)

(b) Individual Cytokinin:

- BA/Kin (0.5-20µM)

(c) Individual Auxin:

- IAA/NAA/2,4D (0.5-10µM)

(d) Combination of Cytokinin+ Cytokinin

- BA (0.5- 20µM) +Kin (0.5- 20µM) in *P oleracea*
- BA (0.5- 10µM) +Kin (0.5- 10µM) in *P grandiflora*

(e) Combination of Cytokinin+ Auxin

- BA (0.5- 20µM)/Kin (0.5- 20µM) + IAA / NAA/2,4D(0.5- 20µM) in *P oleracea*
- BA (0.5- 10µM)/ Kin (0.5- 10µM) + IAA / NAA/2,4D(0.5- 10µM) in *P grandiflora*

Bud Break in *P grandiflora*:

The following components were used for the initiation of in vitro shoots from shootbuds initiated on M.S media supplemented with 10µM BA and 5µM IAA.

- Plant Growth regulator:
    a) Basal media
    b) BA (15- 30 µM)
    c) TDZ (5 µM)
    d) $AdSO_4$ (0.5-5µM)
    e) IAA (2.5 µM)
    f) GA_3 (5 µM)
- Additives
    a) Silver nitrate (5-20 µM)
    b) Coconut water (5%)
    c) Phloroglucinol (5 µM)

(2)Elongation of in vitro shoots in *P oleracea* and *P grandiflora*

A cluster of shoots obtained on the optimized medium for both the plants were transferred to elongation medium which comprised of different hormones and additives

- Plant Growth regulator:

a) Basal Media
b) BA (10- 30 µM)
c) GA_3 (5µM)

- Additives
    a) Phloroglucinol (5µM)
    b) Coconut water (5%).

(3) Rooting of microshoots in *P oleracea*

Elongated shoots (2-3 cms) were transferred to liquid M. S media fortified with 1% sucrose and IBA. Filter paper bridges were introduced in the culture vessels to assist rooting, and the following combinations were tried for *P oleracea*

- Quarter Strength: IBA (1-4 µM)
- Half Strength: IBA (1-4 µM)
- Full Strength: IBA (1-4 µM)

The anatomy of developmental stages of in vitro shoot formation was also studied in *P oleracea*. The explants were inoculated on an optimized medium for regeneration (5µM BA+ 10µM IAA), harvested weekly and fixed in FAA. Sections of 10 – 15 µm were cut with a rotary microtome stained in Hematoxylene and Eocine and mounted in DPX. Photomicrographs were taken with Leica research microscope.

(ii)Regeneration from invitro leaf explants of *P oleracea*

Shoot cultures were also established utilizing in vitro leaf explants on the media which was optimized for in vivo leaf explant. Data was expressed in terms of number of shoots per explants and percent response for regeneration studies.

Callus Cultures in *P oleracea*

Callus cultures were initiated from leaf explants of *P oleracea* on M.S medium supplemented with BA (0.5-10µM) and 2,4D (0.5-10µM) and biomass was calculated for fresh and dry weight. Callus kinetics for biomass on optimized media was also calculated.

(II) Regeneration from nodal explants of *P grandiflora*

The effect of cytokinin (BAP and Kin) was studied on nodal explants of *P grandiflora*.

i) Initiation of in vitro shoots

Single node was taken from the in vivo plant and inoculated on cytokinin rich media

The combinations tested were as follows:

(a) Basal Media (Control)

(b) Cytokinin:

- BA/Kin (0.5-10µM)

(c) Cytokinin+ Cytokinin

- BA (0.5- 10µM) +Kin (0.5- 10µM)

2) Rooting of micro shoots in *P grandiflora*

For rooting half strength M.S media supplemented with 1% sucrose and IBA (2µM) was used.

Shoot Tip Necrosis in nodal cultures of *P grandiflora* was studied with different concentration of $CaCl_2$, (3-30mM), Cagluconate (3-30mM) Boron (0.1-0.4mM). These additives were incorporated in Stock A and Stock B and their effect was recorded in terms of number of shoots per explants and % necrosis.

(B) SECONDARY METABOLITE STUDIES

P oleracea has a high content of fatty acids and the stem of *P grandiflora* is rich in betalains so these two secondary metabolites were targeted in the present investigation.

(I) *P oleracea*– Fatty Acids

Qualitative analysis for the presence of fats was done by Sudan III test and Emulsion test.

Soxhlet extraction of fatty acids was done in petroleum ether for 12 hours. Ten grams of dried material of the following samples were evaluated for the presence of fattyacids,

1) In vivo sample

a) Whole plant
b) In vivo shoot
c) Roots
d) Stem

e) Leaves

2) In vitro samples

a) In vitro shoots of optimized media

b) Callus culture of optimized media

Percentage amount of fat in all these samples was also calculatedas:

Percentage of fat = (W2-W1)/S × 100

W2=weight of the flask+ extracted fat

W1= weight of the flask

S= weight of the sample.

The Fatty acids of the samples were converted to Fatty acid methyl esters by (Weston et al 2008) method. One µl of the sample was injected in Shimaduzu G.C instrument. The column used in the instrument was Supelco Wax-10 (30m×0.25µm×0.25µm) and the detector used was FID.

The fatty acids were identified by comparing the retention time of the sample with that of the standards. The standard used in the study was Supelco37 component FAME mixture, varied concentrations in dichloromethane. Further confirmation of the fatty acids was done by G.C /M.S analysis.

. (II) *P grandiflora* – Betalains

Qualitative analysis of betalains was done by paper chromatography of *P grandiflora* stem and roots of beet root. Dried plant material was extracted with 60% methanol in a Buchner funnel (Kugler et al 2007). The filterate was diluted with McIvaines buffer (pH6) and the content of betaxanthin and betacyanin was determined individually at 480 and 540 nm respectively on UV/ Vis Spectrophotometer. For taking O.D absorbance was set at 0.9nm ≥A≤1. Betalain content for whole plant, stem and leaves was determined by (Cai et al 2005).Ten grams of dried material was extracted for betalains from

(i) In vivo plant

- Whole plant

- Stem
- Leaves

(C)MUTATION STUDIES

The explants used for mutation studies were in vitro leaves harvested from the shoot cultures obtained from in vivo leaf explants in both the *Portulaca* species. The in vitro leaves were treated with a chemical mutagen (EMS)

The following treatment was given:

(i) Ethyl Methane Sulphonate

- 0.1% EMS
- 0.2% EMS
- 0.3% EMS

RESULTS

The present investigation was carried out on three aspects, regeneration studies, secondary metabolite content and the effect of mutagens on the two selected plants.

(A) REGENERATION STUDIES

(I) *P oleracea*

(i) Regeneration from in vivo leaf explants

The in vivo leaf explants were inoculated on a media supplemented with different PGR's and their effect was evaluated.

1) Initiation of in vitro shoots

BA, Kin (cytokinin) and NAA, IAA, 2,4D, IBA (auxins) were taken as the plant growth regulators. These PGR's were used singly as well as in synergistic combinations.

(a) Basal Media (Control)

On the basal media the leaf explants formed1-2 shoots per explants with low percent response.

(b) Cytokinins

(i) BA (0.5- 20µM)

Individual concentration of BA at 5 and 10 µM formed in vitro shoots while increase in concentration lead to decline in number of shoots as well as percent response.

(ii) Kin (0.5- 20µM)

No significant effect of individual concentration of Kin was observed as 1 or 2 in vitro shoots were formed at a high concentration of 15 µM.

(c) Auxins

(i) NAA (0.5- 10µM)

Single concentration of NAA was found to evoke root formation; however at 0.5 µM. roots as well as 1-2 invitro shoots per explants were also formed.

(ii) IAA (0.5- 20µM)

Incorporating IAA in the medium induced roots and shoots formation at 0.5- 10µM concentrations. These invitro shoots were long and stout. Increasing the concentration to 20µM induced only roots.

(iii) 2,4D(0.5- 10µM)

2,4 D is an auxin used for inducing callus formation. At 2.5µM. and 5µM. little white friable callus was formed while no callus formation was observed at 10µM.

(d) Cytokinins+ Cytokinins

(i)BA (0.5-20µM) and Kin (0.5- 20µM):

Direct regeneration from leaf explants was obtained in this combination. Optimum number of in vitro shoots was obtained at 5µM BA and 10µM Kin. Further increase in concentration of BA and Kin formed callus and decline in number of shoots/explants.

(e) Cytokinins +Auxin

(i) BA(0.5-10µM) and IAA (0.5-10µM):

Low concentration of BA (0.5 µM- 2.5µM.) with increasing concentration of IAA (0.5.-10 µM) formed roots along with callus. Optimum response is obtained at 2.5 µM BA and 2.5µM IAA which promotes highest number of roots per explant and percent response for roots is 100%. Callus formation was observed at high levels of BA (10µM.) and IAA (10µM.)

(ii)BA(0.5-10 µM) and NAA (0.5-10 µM):

Predominantly roots were formed with 0.5 µM and 2.5µM BA and increasing concentration of NAA. Increase in concentration of BA (5-10µM) along with increasing concentration of NAA formed profuse callus and roots.

(iii) BA (0.5-10µM) and 2,4 D(0.5-10µM):

Increasing concentration of BA from 0.5 – 10µM along with 0.5µM 2,4D gave myriad response. These combinations were noted for the formation of root, shoots as well as callus. The callus was white and friable at 0.5µM BA + 0.5µM 2,4D and 2.5µM BA with 0.5µM 2,4D which turned to green and nodular at higher BA concentrations. Increase in BA along with 2,4D formed white friable callus. Fresh weight and dry weight of callus confirmed 2.5µM BA and 2.5µM2,4 D as an optimum concentration for the formation of callus.

(iv)Kin (0.5-10µM) and IAA (0.5-10µM.**)**

Low levels of Kin (0.5-2.5µM.) along with increasing concentration of IAA formed roots with negligible callus formation. At 5µM Kin and increasing concentration of IAA (0.5-10µM.) moderate callus and roots were obtained. No response was observed at higher concentration of Kin (10µM) and IAA.

(v)Kin (0.5-10µM) and NAA (0.5-10µM)

The number of roots per explants was less as compared to BA and NAA combinations. Besides this callus formed was also moderate in amount. Optimum root formation per explants (4.33) and

moderate callus was obtained at 5 µM BA and 10µM NAA. The callus was friable and slightly yellow.

(vi) Kin and 2,4 D (0.5-10µM)

Increasing concentration of Kin (0.5- 10µM.) along with 0.5µM 2,4 D formed roots shoots and negligible callus. Further increase in concentration of 2,4 D and Kin formed moderate white friable callus.

2) Elongation Medium

Elongation of the in vitro shoot clusters was achieved on basal media supplemented with 5% Coconut water.

3) Rooting of micro shoots

Half strength liquid M.S media supplemented with 1% sucrose and 3µM IBA was optimized for rooting.

The anatomy of developmental stages of invitro shoot formation was also studied. The onset of shoot bud differentiation took place from mesophyll cells. Promeristemoids were formed indicating shoot bud formation. Apical meristem with shoot primordium was visible by 4 week.

Callus Cultures

Callus biomass growth kinetics was initiated on an optimized medium for callus (2.5µM BA and 2.5µM 2,4D). The graph plotted (Callus growth index versus time) revealed 7^{th} week as the time period where the growth of the callus peaked after which there was a steady decline.

(II) *P grandiflora*

Regeneration in *P grandiflora* was obtained from leaf and nodal explants.

(a) Regeneration from in vivo leaf explants

The effect of different PGR's was evaluated on the growth and morphogenesis.

Cytokinins

(i) BA (0.5- 20µM)

At 5—20µM very little white compact callus was formed.

(ii) Kin(0.5- 20µM)

Individual concentration of Kin also gave poor response. At 2.5 -5µM very little callus with 30% response was formed.

Auxins

(i) NAA (0.5- 20µM)

This auxin at 5—10µM formed heterogeneous white, green with tinges of pink callus. The callus was friable and very little.

(ii) (IAA (0.5- 20µM)

No Response

(iii) 2,4D(0.5- 10µM)

No response was obtained in these combinations.

Cytokinin+ Cytokinin

(iv) BA and Kin (0.5- 20µM)

The leaf explants inoculated on these combinations formed one or two buds per explants which formed roots.

Cytokinins and Auxin

(i) BA (0.5- 10µM) and IAA (0.5-10µM):

Diverse results were obtained in this set of PGR combination for *P grandiflora*. Callus and shoot buds were obtained in these combinations. At 0.5µM and 2.5µM IAA white green callus was formed however with increase in IAA and BA this type of callus was replaced by a

heterogeneous mass of white, green and red nodular callus. Bud break was not observed in these shoot buds even after 8 weeks. At 10µM BA and 5µM Kin cluster of shoots was formed with only 10% success so this combination was selected for further manipulations.

(ii) BA (0.5- 10µM) and NAA (0.5- 10 µM)

Shoot buds and callus was formed at 2.5 µM BA and increasing concentration of NAA. The callus was friable white and slightly red. The number of shoot buds decreased at higher concentrations of BA and the callus turned green white.

(iii) BA (0.5- 10µM) and 2,4 D(0.5- 10µM)

This synergistic combination of cytokinin and auxin also formed callus however diversity in texture and type of callus was noted. 2.5µM. BA and increasing concentration 2,4 D formed white friable callus. At 5 BA and varying concentration of 2,4 D (0.5-10µM.) white friable and green compact both type of callus was formed. Increasing the concentration to 10µM. BA formed green compact callus.

(iv) Kin (0.5- 10µM)and IAA (0.5- 10µM)

(2.5 µM) Kin and IAA (0.5-10 µM) formed callus after 3 weeks with shoot buds. The callus was white red and friable. Similar response was obtained at higher concentration of Kin (5 µM Kin – 10 µM Kin) and increasing concentration of IAA. Thus shoot buds formed in these combinations was not accompanied by bud break.

(v) Kin (0.5- 10µM)and NAA(0.5- 10 µM)

Kin at 2.5µM and increasing concentration of NAA formed roots and callus. The callus was green white friable type. At higher concentration of Kin and NAA moderate greenish white callus both nodular and friable was formed.

(vi) Kin (0.5- 10µM) and 2,4 D(0.5- 10µM)

No response was observed at 0.5µM Kin and increasing concentration of 2,4 D. Further increase in Kin and 2,4 D concentrations formed green friable callus with poor proliferation.

Bud Break media:

For the formation of in vitro shoots many additives were tried such as Phloroglucinol, GA_3, coconut water, Silver nitrate and TDZ and basal media which did not give promising results.

First nodal leaf lead to prolific initiation of in vitro shoots from the shoot buds indicating that the age of the explants also plays an important role in in vitro morphogenesis. The initiated shoots were transferred on to multiplication media of 20µM. BA where a cluster of in vitro shoots were formed.

Elongation media

Coconut water and GA_3 were employed for elongation of shoots of which GA_3 formed elongated shoots. Optimum concentration for elongation of micro shoots with GA_3 is being standardized.

(b) Regeneration in nodal explants:

Regeneration in nodal cultures was initiated in cytokinin rich medium.

(a) Basal Media (Control)

No response was obtained on basal media.

(b) Cytokinin:

- BA/Kin (0.5-10µM)
  Single concentration of BAP had a significant effect on multiple shoot formation as compared to individual concentration of Kin

(c) Cytokinin+ Cytokinin

- BA (0.5- 10µM) +Kin (0.5- 10µM)
  Optimum number of multiple shoots was formed at 8µM Kin and 2µM BAP

2)Rooting of microshoots

Rooting was achieved at half strength M.S media supplemented with 1% sucrose and IBA (2µM).

The multiple shoots formed from nodal cultures were accompanied with shoot tip necrosis. $CaCl_2$ at 18 mM was suitable to reduce STN.

(B)SECONDARY METABOLITE STUDIES

As a defense mode plants synthesize secondary metabolites which can be used as medicines, food additives and industrially important products. The present study investigates the presence of two secondary metabolites fatty acids and betalains in *P oleracea* and *P grandiflora* respectively.

(I) *P oleracea* (Fatty Acids)

Qualitative analysis of the plant samples revealed that except roots of *P oleracea* all the other samples tested positive for the presence of Fatty acids. Hence quantitative analysis using gas chromatography was done on these samples only. Maximum percentage of fat was found in vivo leaves followed by whole plant and stem. Interestingly in vivo shoot and in vitro shoot almost had the similar percent of fatty acids implying that in vitro shoots could also be a good source of fatty acids.

After G.C/M.S analysis the following fatty acids were detected in the plant samples -:

Chemical Name	**Common Name**	**Stem**	**Leaf**	**Invivo Plants**	**Invitro Plants**	**Callus**
Methyl Palmitate	Palmitic Acid	✓	✓	✓	✓	✓
Methyl Stereate	Stearic Acid	✓	✓	✓	-	-
Methyl Linoleate	Linoleic Acid	✓	✓	✓	✓	✓
Methyl Linolenate	Alpha Linolenic Acid	✓	✓	✓	✓	✓
Pentadecanoic acid	Pentadecyclic Acid	✓	✓	✓	✓	✓
Methyl decosanoate	Docosanoic Acid	✓	✓	✓	✓	✓
Methyl eicosanoate	Arachidic acid	-	-	-	-	✓
Methyl pentacosanoate	Pentacosanoic Acid	✓	✓	✓	-	✓
Methyl hexacosanoate	Hexacosanoic Acid	✓	✓	✓	✓	-

Further analysis and quantification is being carried out on these samples.

(II) *P grandiflora* (Betalains)

Qualitative analysis was done by paper chromatography of dried material of *P grandiflora* stem and beetroot. The Rf value for both the material was 0.3 indicating the presence of betalains in *P grandiflora*. The extraction of betalain from whole plant, stem, leaves and root was carried out.

Maximum betalains was reported in stem followed by whole plant, whereas negligible betalain was detected in leaves and it was completelyabsent in roots.

(C) MUTATION STUDIES

Genetic variability can be induced by introducing mutagens in biological samples. The quality and quantity of secondary metabolite can be influenced by the treatment of plant materials from these physical and chemical mutagens. In the present study *P oleracea* and *P grandiflora* will be treated with mutagens and their effects would be assessed.

(I) *P oleracea*

The in vitro leaf explants was treated with different concentration of EMS (0.1-0.4%). Preliminary studies indicate that 0.1% and 0.2% EMS at 1 hour of treatment formed in vitro shoots. Concentration beyond 0.4% showed visible lesion on the explants after treatment. Plants were formed where the leaves turned acicular instead of ovate.

In vitro leaf explants was also treated with EMS 0.1-0.4% for 1 hour and inoculated on an optimized callus culture media (2.5µM BA+2.5µM 2,4D). Studies indicated that maximum biomass was formed at 0.2% after which there was a steady decline.

(II) *P grandiflora*

Similar studies are initiated and results are awaited for *P grandiflora*

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