EXPERIMENTAL RESULTS

Costus pictus is an important medicinal plant. This plant grows naturally in southern part of our country. Natural propagation of this plant in conditions of Marathwada region of Maharashtra state is not possible. Hence the in vitro propagation techniques have been advocated for its multiplication. This plant contains palmitic acid which is undesirable for its utility as a medicine and hence efforts were made for in vitro mutation induction and biochemical analysis to evaluate its medicinal potential. Results obtained during the present investigation are presented in three parts, and they comprise:

- Evaluation of in vitro propagation,
- In vitro mutagenesis,
- Phytochemical studies.

Evaluation of in vitro propagation techniques

Plants were collected from nurseries located at Coimbatore and authenticated at BAMU herbarium, Aurangabad. Plants were grown and maintained in green house. To standardize efficient protocol for propagation of Costus pictus, all experiments were carried out in the Tissue Culture Laboratory, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad.
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Effect of HgCl$_2$ on explant survival of *Costus pictus* and control of contamination (Table 4)

Plant material was collected from elite plant grown in green house, located in Botanical garden at Botany Department of Dr. BAMU, Aurangabad. Explants were surface sterilized with mercuric chloride (HgCl$_2$). Standard protocol of surface sterilizing agents for *Costus pictus* was standardized through trial and error method. The leaves, nodal segments and shoot tip of *Costus pictus* were surface sterilized with different concentrations of mercuric chloride (0.05-0.5%). Data for survival of explants and contamination were recorded after 30 days of inoculation as shown in table 4.

The healthy plant material was washed with running tap water followed by sterile distilled water. Explants were surface sterilized with 0.05 to 1.0% mercuric chloride. In case of leaf explants the contact periods of sterilizing agent were for three and five minutes. In case of nodal segment and shoot tip explants, it ranged from 0.1-0.5% for three minutes. Leaf explant was free from contamination at 0.3% concentration of HgCl$_2$ but explants turned white and the survival percentage was 10%. Maximum survival rate was achieved at 0.1% concentration of HgCl$_2$ for 3 minutes, but contamination was not controlled completely and therefore duration of sterilization was increased upto five minutes, which was found suitable.

For nodal and shoot tip explants, lower concentration of mercuric chloride was not suitable but with an increase in concentration above 0.3%, the survival
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percentage got decreased. Significant effect on percentage of survival has been recorded at 0.3% HgCl₂.

**Induction of Callus:**

In the present investigation, MS medium was fortified with various concentrations and combinations of 2, 4-D, IAA, NAA, KIN and BAP. The induction of callus was noticed quite very well. Different explants were tried which comprised leaf, nodal segment and shoot tip of juvenile plantlet. Cultures were observed at regular intervals for frequency of callus induction, texture of callus, colour of callus and induction of somatic embryos.

**Influence of 2, 4-D on callus induction in Costus pictus** (Table 5 and photo plate 1)

During present experiment, explants like leaf, nodal segments and shoot tips were excised from juvenile plants grown in green house. MS medium with basal salt mixtures was utilized for induction of callus from above mentioned explants with different concentrations of 2, 4-D ranging from 0.5- 2.5 mg/L. It revealed that 2, 4-D alone facilitated the induction of callus at high or low level in MS medium as indicated in table 5 and photo plate 1.

Callus was noticed using leaf as an explant. Low concentration of 2, 4-D, 0.5 mg/L did not show any response as regards proliferation, whereas 1.0 mg/L concentration showed swelling of explants, while maximum callus induction could be noticed with 2, 4-D, 1.5 mg/L. Increase in concentration of 2, 4-D subsequently decreased the frequency of callus formation. Leaf derived callus was friable and
whitish in colour. The maximum frequency of callus induction was recorded at 1.0 mg/L of 2, 4-D using nodal segment as an explant. The moderate frequency of callus induction has been recorded at 1.5 mg/L and 2.0 mg/L concentrations of 2, 4-D. All these concentrations could induce white yellow friable callus in second week. Somatic embryogenesis could be recorded after 4 weeks in nodal segment derived callus.

The MS medium without growth regulators did not favor induction of callus from leaf, nodal segments and shoot tip as explant, but when media were supplemented with various concentrations of 2, 4-D, it facilitated friable callus formation with induction of somatic embryos. Maximum frequencies of callus induction and induction of somatic embryogenesis have been recorded from nodal segment compared with other explants.

**Effect of 2, 4-D alongwith BAP on callus induction:** (Table 6 and photo plate 2)

Present experiment was aimed to observe response of 2, 4-D and BAP on various explants for callus induction. Different combinations of growth regulators were tried with previously developed *in vitro* culture as a source of explants. Observations were recorded after three to four weeks and tabulated in table 6.

The observations recorded in this table clearly indicate poor callus induction from 2, 4-D in combination with different concentrations of BAP at 1.0, 1.5 and 2.0 mg/L supplemented MS medium. Different explants tried were leaf, nodal segment and shoot tip. Moderate frequency of callus induction has been recorded at 2.0 mg/L of 2, 4-D in combination with 1.0 mg/L of BAP. It was
found that, the highest frequency of callus could be recorded with nodal segment compared with other explants. A poor range of callus has been induced from 1.5 mg/L of 2, 4-D in combination with 1.0 mg/L of BAP using leaf explant, while 2.0 mg/L and 1.5 mg/L of 2, 4-D in combination with 1.0 mg/L of BAP were found best using nodal segment and shoot tip as explants, respectively.

The lower concentration of 2, 4-D in combination with BAP did not show significant effect on callus induction in all the above three explants. MS medium incorporated with minimum concentration of 2, 4-D and BAP revealed swelling of explants followed by death of explants. The nodal segment as an explant resulted in swelling and after three weeks induction of shoot could be recorded.

**Effect of IAA in combination with BAP on callus induction** (Table 6)

To develop standard protocol for induction of callus in *C. pictus*, an experimental set up was made to observe the effect of different types of explants and various concentrations of IAA, 0.5, 1.0 and 1.5 mg/L in combination with 0.5, 1.0 and 1.5 mg/L of BAP. The excised leaf, nodal segment and shoot tip region were isolated from *in vitro* grown plantlets of six to seven weeks old cultures and used as explants in the experimental set up. Higher concentrations of growth regulators such as 1.0 and 1.5 mg/L of IAA and BAP in combinations have shown swelling of explant after three weeks but failed to induce the callus. All other combinations of IAA and BAP were not influencing adequately induction of callus.
CAULOGENESIS

Regeneration of shoot from callus (Table 7 and photo plate 3)

Experiments on caulogenesis included regeneration of shoot from callus derived from various explants like leaf, nodal segment and shoot tip on MS medium. It also comprised 0.5 mg/L of IAA in combination with different concentrations of BAP ranging from 0.5-2.0 mg/L. Maximum regeneration of shoot percentage was recorded on MS medium incorporated with 0.5 mg/L of IAA in combination with 1.5 mg/L of BAP which showed 30% regeneration of shoot using leaf explants, while 1.0 mg/L and 2.0 mg/L of BAP showed moderate percentage of regeneration of shoot.

Maximum regeneration percentage of shoot has been noticed from nodal segment derived callus, subcultured on MS medium incorporated with IAA 0.5 mg/L in combination with 1.5 mg/L of BAP, and it was 50%. Moderate shoot regeneration could be recorded on 1.0 mg/L of BAP in combination with 0.5 mg/L IAA. Whereas callus derived from shoot tip demonstrated poor regeneration of shoot percentage on higher concentration of BAP 1.5 and 2.0 mg/L in combination with IAA.

Direct shoot regeneration

The present studies were undertaken to standardize protocol for direct regeneration of shoot from various explants viz. leaf, nodal segment and shoot tip of C. pictus. Rate of multiplication was higher in this method of in vitro
propagation. Similarly production of larger number of identical plants could be achieved through this technique.

**Effect of Cytokinins like BAP /KIN on caulogenesis:** (Table 8 and photo plate 4)

The experimental set up was tried to study the effect of different concentrations of BAP and KIN in MS medium alongwith IAA, using leaf and nodal segment as explants. Initially, different explants like leaf and nodal segment were inoculated on MS medium fortified with 0.5 mg/L of IAA alongwith various concentrations of BAP ranging from 0.5-3.5 mg/L.

Results showed 13.33 - 93.33% of shoot induction. Leaf explants showed maximum shoot regeneration at 0.5 mg/L of IAA with 2.5 mg/L and 3.0 mg/L of BAP facilitating 73.33% and 66.66% shoot regeneration, respectively. Whereas moderate shoot regeneration could be recorded at 1.5 mg/L and 2.0 mg/L of BAP and it was 53.33% of shoot regeneration. Lower concentration of BAP and IAA, (0.5 mg/L) did not show detectable change in the explant.

Effect of various concentrations of KIN in combination with 0.5 mg/L of IAA was tested. Maximum shoot induction was recorded at 4.0 mg/L of KIN alongwith 0.5 mg/L of IAA, which was 40%. Whereas other concentrations of growth regulators showed 20.00% to 33.33% of shoot induction.

To standardize the direct regeneration from nodal segments, MS medium was supplemented with 0.5 mg/L of IAA in combination with various concentrations of BAP and KIN. Higher numbers of shoot induction were
achieved at 3.0 mg/L of BAP. Shoot development was also achieved at 2.5 mg/L of BAP in combination with IAA. Increase in shoot induction was recorded as concentration of BAP increased from 0.5 to 3.0 mg/L in combination with 0.5 mg/L with IAA. Lower concentration of BAP in combination with IAA was in favor of lower rate of shoot induction from nodal segments.

MS medium along with 0.5 mg/L of IAA with various concentrations of KIN ranging from 1.0-5.0 mg/L facilitated 13.33% to 66.66% of shoot induction using nodal segment as explant. Average shoot induction was achieved at 3.0 mg/L and 5.0 mg/L of KIN along with 0.5 mg/L of IAA, which was 53.33% and 60.00%. Maximum shoot induction and highest number of shoots were recorded on MS medium containing 4.0 mg/L of KIN along with 0.5 mg/L of IAA as indicated in Table 8 and photo plate 4.

**Effect of BAP and IAA on multiple shoot formation** (Table 9)

Effect of BAP 2.5 mg/L and 3.0 mg/L along with IAA ranging from 0.5-3.0 mg/L in MS medium using leaf and nodal segments was tried for induction of multiple shoot formation. The results revealed maximum frequency of multiple shoot formation at lower concentration of IAA, 0.5 mg/L and BAP 2.5 mg/L using leaf as an explant, which comprised 73.33% and 2.86±0.445 number of shoots per explant. Increase in concentration of IAA decreased number of shoots as shown in table 9. Similar results were obtained at 3.0 mg/L of BAP along with 0.5 mg/L of IAA using leaf explant. Most suitable concentration recorded was 0.5 mg/L of
IAA in combination with BAP 3.0 mg/L, which showed 66.66% of shoot regeneration and 1.80±0.380 number of shoots per explant.

To achieve multiple shoot induction from nodal segment explants, they were inoculated on MS medium containing 2.5 mg/L and 3.0 mg/L of BAP alongwith various concentrations of IAA. The observations revealed induction of maximum number of shoots on MS medium supplemented with 3.0 mg/L of BAP in combination with 0.5 mg/L IAA which was 93.33% with 3.86±0.336 number of shoots per explant. Whereas the average number of shoot induction could be achieved on 1.0, 1.5 and 2.0 mg/L of IAA alongwith of 3.0 mg/L of BAP. Increase in concentration of IAA 0.5-3.0 mg/L with 3.0 mg/L of BAP decreased the number and percentage of shoot regeneration. BAP 2.5 mg/L in combination with various concentrations of IAA was tested for multiple shoot. Maximum shoot induction (86.66%) was achieved at lower concentration of IAA which comprised 0.5 mg/L. Higher concentration of IAA showed rhizogenesis as indicated in photo plate 5.

**Effect of BAP and NAA on induction of multiple shoots** (Table 10 and photo plate 6)

Effect of BAP and NAA was also studied with various concentrations in MS medium using leaf and nodal segment as explants. Maximum number of shoot regeneration was achieved at BAP 2.5 mg/L with NAA 0.5 mg/L which showed 66.66 percent of shoot regeneration with 1.86±0.215 number of shoot with leaf explant. Increased concentration of NAA decreased the shoot regeneration
percentage and number of shoots per explant. Whereas 3.0 mg/L of BAP in combination with NAA showed 1.53±0.255 number of shoots per leaf explant and 73.33% of shoot regeneration.

Nodal segments were inoculated on MS medium with 3.0 mg/L of BAP and 0.5 mg/L of NAA. This combination was found most effective which showed 100% shoot regeneration with 3.86±0.165 shoots per nodal segment explant. Whereas 2.5 mg/L of BAP in combination with 0.5 mg/L of NAA showed 93.33% of shoot regeneration with 3.66±0.360 shoots per explant. Both concentrations of BAP 2.5 mg/L and 3.0 mg/L in combination with 1.0 mg/L of NAA revealed moderate shoot regeneration and number of shoots per explant. Higher concentration did not favor maximum shoot induction and the number of shoots also was less. BAP at 2.5 mg/L and 3.0 mg/L in combination with lower concentration of NAA was found most suitable for induction of multiple shoots from leaf and nodal segment as explants as listed in Table 10.

**Effect of KIN and IAA on induction of multiple shoots** (Table 11 and photo plate 7)

Effect of KIN in combination with IAA was tried on MS medium with leaf and nodal segment as explants to achieve multiple shoots. The results revealed that maximum shoot regeneration percentage could be recorded as 70.00% with 1.50±0.372 number of shoots using leaf as an explant, on MS medium incorporated with 4.0 mg/L of KIN in combination with 0.5 mg/L of IAA. The combination of 5.0 mg/L of KIN along with 0.5 mg/L of IAA showed maximum
regeneration percentage, which could be recorded as 70.00% with 1.40±0.371 number of shoots. While the increased concentrations of IAA ranging from 0.5-3.0 mg/L, led to decrease in regeneration percentage and number of shoots per explant as indicated in table 7.

Effects were analyzed at 4.0 and 5.0 mg/L of KIN in combination with 0.5-3.0 mg/L of IAA on nodal segment as explant for multiple shoot induction. It revealed 60-90 % of shoot induction with 1.20±0.326 to 2.40±0.305 number of shoots per explant. Most suitable concentration recorded was 4.0 mg/L of KIN in combination with 0.5 mg/L of IAA with 90 % of shoot induction and 2.40±0.305 number of shoots per explant. While 5.0 mg/L of KIN along with 0.5 mg/L of IAA showed 80.00% of shoot induction and 2.10±0.378 number of shoots per explant. Higher concentration of IAA showed maximum root induction but shoot induction and number of shoots per explant however decreased.

**Effect of KIN in combination with NAA and 2, 4-D on Multiple shoot induction.** (Table 12)

To achieve the multiple shoot induction from leaf and nodal segment explants, various concentrations of NAA and 2, 4-D along with 4.0 mg/L and 5.0 mg/L of KIN were tried. The observations revealed that the leaf explant showed 60.00-73.33% shoot induction and 1.06±0.248 - 1.60±0.289 number of shoots per explant. Maximum shoot induction percentage was 73.33 with 1.60±0.289 number of shoots per explant recorded at 4.0 mg/L of KIN along with 0.5 mg/L of NAA. Whereas 1.0 and 1.5 mg/L of NAA showed moderate shoot induction percentage
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and number of shoots per explant. Leaf explant with respect to 5.0 mg/L of KIN and 0.5-2.5 mg/L of NAA revealed similar kind of results with 4.0 mg/L of KIN. Maximum shoot induction has been recorded at 0.5 and 1.0 mg/L of NAA in combination with 5.0 mg/L of KIN which was 73.33 % and 1.53±0.321 and 1.40±0.289 number of shoots per explant, respectively.

Nodal segment showed better results than leaf explant. 4.0 mg/L of KIN in combination with 0.5-2.5 mg/L of NAA showed 80.00- 93.33% of shoot induction and 1.86±0.273 to 2.53±0.236 number of shoots per explant. Maximum shoot induction and higher number of shoots per explant have been recorded at 0.5 and 1.0 mg/L of NAA in combination with 4.0 mg/L of KIN, which comprised 93.33% of shoot induction and 2.53±0.236 and 2.40±0.213 number of shoots per explant, respectively. While rest of the concentrations indicated moderate shoot induction percentage and number of shoots per explant. 5.0 mg/L of KIN in combination with 0.5-2.5 mg/L of NAA showed 73.33-86.66% of shoot induction and 1.40±0.235 to 2.06±0.248 number of shoots per explant. Most suitable concentration recorded was 0.5 mg/L of NAA alongwith 5.0 mg/L of KIN with 86.66% of shoot induction and 2.06±0.248 number of shoots per explant. Moderate shoot induction and number of shoots have been achieved at 1.0 mg/L and1.5 mg/L of NAA in combination with KIN. Leaf and nodal segment induced multiple shoots at lower concentration of NAA in combination with 4.0 mg/L and 5.0 mg/L of KIN. The increased concentrations of NAA led to decreased shoot
induction percentage and number of shoots per explant as shown in table 12 and photo plate 8.

Effects of various concentrations of 2, 4-D ranging from 0.5-2.5 mg/L in combination with KIN 4.0 mg/L and 5.0 mg/L were analyzed for multiple shoot induction from leaf and nodal segment explants. Observations revealed that 4.0 mg/L of KIN in combination with 2, 4-D (0.5 to 2.5 mg/L) induced 46.66-80.00% of shoot regeneration with 0.66±0.186-1.20±0.200 number of shoots per explant. Maximum percentage of shoot induction, which was 80.00% with 1.20±0.200 number of shoots per explant at 4.0 mg/L of KIN along with 0.5 mg/L of 2, 4-D could be noticed by using leaf as an explant. 1.0 mg/L and 1.5 mg/L of 2, 4-D showed moderate shoot induction and number of shoots per explant. Higher concentration of 2, 4-D in combination with KIN 4.0 mg/L facilitated poor callus induction and the shoot induction could be recorded only after two weeks. While 5.0 mg/L of KIN in combination with 0.5-2.5 mg/L of 2, 4-D, showed 60-80% of shoot induction and 0.80±0.200 to 1.13±0.191 number of shoots per explant. Most suitable concentration of 2, 4-D recorded was 0.5 mg/L along with 5.0 mg/L of KIN, which showed 80.00% of shoot induction and 1.13±0.191 number of shoots per explant. Whereas, with increase in the concentration of 2, 4-D, there was a subsequent decrease in shoot induction and number of shoots per explant. Higher concentration of 2, 4-D helped shoot induction in *Costus* along with moderate callus induction.
Nodal segment explant was also tried for induction of multiple shoots by using various concentrations of 2, 4-D along with KIN incorporated in MS medium. Observations revealed that 4.0 mg/L of KIN along with 0.5 and 1.0 mg/L of 2, 4-D showed maximum shoot induction which was 80.00% with 2.00±0.292 and 1.86±0.273 shoots per explant. Similar kinds of results were recorded at 5.0 mg/L of KIN. Increase in concentration of 2, 4-D resulted in decreased shoot induction percentage and number of shoots per explant. Whereas, the higher concentration supported growth of callus alongwith shoot.

**Rhizogenesis in vitro**

**Effect of IAA and NAA on in vitro root induction** (Table 13 and photo plate10)

Experiments were conducted to check the effect of IAA and NAA for the induction of root from cultures on MS medium. In first part of experiment the MS medium fortified with 1.0 – 5.0 mg/L of IAA was tried. It has been noticed that there was 60 – 100% of root induction. Observations revealed that initiation of regular fibrous roots induction as well as large number of adventitious roots became possible from nodal part of shoots. The maximum root induction was recorded at 1.0 and 2.0 mg/L of IAA, which was 100%. The average length of fibrous roots 11.14±0.128 cm and 13.14±0.258 cm, while that of adventitious roots 15.6±0.234 cm and 13.42±0.142 cm, respectively could be noted. Whereas 3.0 and 4.0 mg/L of IAA recorded moderate root induction to be 80.00% with length of fibrous roots as 8.88±0.385 cm and 7.66±0.067 cm while those of adventitious roots 12.16±0.120 cm and 10.6±0.122 cm, respectively. Higher
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concentration of IAA 5.0 mg/L showed decrease in root induction percentage, which was found to be 60.00% with length of fibrous root 7.48±0.106 cm and of adventitious roots 10.3±0.284 cm, respectively.

MS medium incorporated with 1.0 – 5.0 mg/L of NAA revealed 60 – 80% of root induction. Most suitable concentrations recorded were 1.0 mg/L and 2.0 mg/L of NAA, which showed 80.00% of root inductions with length of fibrous roots were 10.54±0.132 cm and 12.6±0.100 cm while the length of adventitious roots 13.88±0.305 cm and 12.76±0.143 cm, respectively. 3.0 mg/L to 4.0 mg/L of NAA showed moderate root induction which was 80.00% and the length of fibrous roots recorded were 7.68±0.174 cm and 7.12±0.128 cm while the values of 11.36±0.211 cm and 9.70±0.151 cm comprised the lengths of adventitious roots, respectively. Highest concentration of IAA 5.0 mg/L facilitated 60.00% of root induction with lengths of fibrous root 6.84±0.177 cm and 8.14±0.267 cm as length of adventitious roots, as shown in table 13.

**Hardening of plantlets grown in vitro** (Table14 and photo plate 11)

The well rooted *in vitro* grown cultures were obtained from MS medium supplemented with 1.0 and 2.0 mg/L of IAA/NAA. These *in vitro* grown cultures were supposed to go through the stage of acclimatization with external environment to grow in field. Therefore well rooted culture bottles after 3 weeks were transferred from culture room to the external environment at regular intervals to make plantlet adjust with natural conditions. Survival percentage was
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calculated, which was found to be 100.00% in the first stage of hardening of *in vitro* grown plantlets for a week.

Second step of hardening comprised the removal of microshoots from culture bottles, and their thorough washing in tap water to remove the media and solidifying agent clerigel from the roots carefully. These microshoots were then transferred to plastic cups containing coco peat, for 5 to 6 weeks of period for well hardening of roots. Coco peat holds water for longer period and it also keeps proper aeration. This step was achieved in polyhouse only carrying controlled conditions and adequate humidity was maintained which led to 80.00% of survival of *in vitro* grown plants developed during the present work.

After two weeks of period again these microshoots were transferred to different pots carrying 1:1 ratio of soil: FYM (farm yard manure). They were allowed to absorb nutrients through their roots. Microshoots were allowed to grow for 7th, 8th and 9th weeks in polyhouse only. 80.00% of survival rate could be recorded as shown in table 14 and photo plate 11. These microshoots were transferred to pots containing soil for two weeks and this step showed 80.00% of survival of the cultures. After 11 weeks, these microshoots were ready to grow in field with external conditions. After all these steps the microshoots were well adapted to external environment. Nearly 60.00% of the plantlet hardening was found successful during the present work in *C. pictus*.  

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B. IN VITRO MUTAGENESIS

*Costus pictus* is denoted as insulin plant. During therapy it revealed that presence of palmitic acid in leaves decreases the absorption of metabolites. To reduce the percentage of palmitic acid, it was decided to go for mutagenesis *in vitro*. Effects of various concentrations of mutagens like SA and EMS on morphological and phytochemical properties of *C. pictus* were studied. The *in vitro* mutagenesis experiments were carried out by addition of chemical mutagens directly in the MS medium. Observations were made at regular intervals.

**Effect of mutagens on sprouting of bud from nodal segment under *in vitro* conditions** (Table 15 and photo plate 12)

Sprouting of bud from nodal segments was tested with different concentrations of SA and EMS incorporated in MS medium under *in vitro* conditions. Nodal segments used as explant were collected from the previously *in vitro* raised cultures and aseptically inoculated on MS medium supplemented with 0.5 mg/L of IAA in combination with 3.0 mg/L of BAP and incorporated with various concentrations of SA (0.01, 0.02 and 0.03%) and EMS (0.05, 0.10 and 0.15%). After aseptic inoculation the culture bottles were transferred to culture rooms in controlled condition. Observations were made at regular intervals shown in table 15.

Table 15 would show the effects of mutagens on sprouting of buds of nodal segments which decreased as compared to control. Maximum percentage of sprouting of bud 80.00% was achieved at 0.10% EMS with 2.2±0.374 number of
shoots per explant with the mean length of shoot being 12.62±0.177 cm. However 0.15% and 0.05% EMS showed moderate percentage of sprouting of bud which was 60.00% with 1.8±0.489 and 1.6±0.400 number of shoots per explant with the mean length of the shoot being 14.36±0.479 cm and 8.38±0.193 cm, respectively. SA affected the sprouting of bud at higher levels, where sprouting of bud percentage decreased with an increase in concentration of SA (0.01 -0.03%). The values were found to be 60.00-20.00 % using nodal segment as explant. Maximum percentage of sprouting of bud was achieved at 0.01% of SA with 1.2±0.583 number of shoots having mean length of shoot as 2.84±1.16 cm. Whereas, different concentrations (0.02 and 0.03%) of SA showed less percentage of sprouting of buds, which was 20.00% only, with 0.6±0.400 and 0.4±0.400 number of shoots per explant as shown in photo plate12.

**Induction of mutation in terms of morphological mutants** (Table16 and Photo plate 13)

*In vitro* mutagenesis was tried through inclusion of mutagens in MS medium as mentioned above. Sprouting of buds from nodal segment was recorded. After three to four weeks the plantlets grown from buds were showing variability compared with control plants. These variations could be recorded in terms of long shoot, dwarf shoot, broad stem, thin stem and change in lamina of leaf as shown in table 16.

It is clear from table 16 that, different concentrations of mutagens under *in vitro* led to a wide variety of morphological changes in seedlings. The
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morphological variations revealed differences from the control culture and included long shoot, dwarf shoot, broad stem, thin stem and change in lamina of leaf. The highest mean number and percentage of variants obtained was shown by 0.10% EMS treatment given to nodal segment. Similarly 0.01% treatment of SA induced maximum number and percentage of different morphological alterations (0.80). The leaf morphological variations (change in leaf lamina) were detected earlier after the bud sprouting in the fifth week. These was followed by variants like long shoot, dwarf shoot, broad stem and thin stem which could be identified in the sixth week. The increase in concentrations of both the mutagens (EMS and SA) decreased the proliferation, alteration and variation at culture level.

Studies in in vitro M2 generation

The in vitro M2 generation was raised from nodal segments of C. pictus, especially such segments which were excised and isolated from in vitro grown seedlings raised through mutagenic treatments. Both the excised segments were inoculated on previously tested and standardized shoot regeneration MS medium. In M2 generation, studies were carried out on frequency of chlorophyll mutations, besides their spectrum. Alongwith these studies, the M2 generation was screened for different morphological variants and their frequency values have been recorded in table 17.

Effect of mutagens on in vitro morphological variation in M2 generation

(Table17 and photo plate 14)
Effect of mutagens on \textit{in vitro} raised M$_2$ generation was studied with reference to morphological variations. \textit{In vitro} raised cultures showed morphological variability in stem, shoot and leaf part. Data were collected for mean number of variations per hundred segments grown per plant part as compared to control and recorded in Table 17 (Plate 14 and graph 12).

It is clear from table 17 that, low concentration of mutagens (EMS 0.10\% and SA 0.01\%) from nodal segment derived \textit{in vitro} cultures, developed maximum mean number of morphological variations (3.40 and 1.40). While the higher concentration of mutagens (EMS 0.15\% and SA 0.02 and 0.03\%) proved less effective in regard to alteration of morphological characters (2.20 and 0.60 and 0.40). Majority of induced variations were noted in morphological characters such as long shoot, dwarf shoot and broad stem from nodal segment explant.

Table 17 would reveal that long shoots, dwarf shoots, broad stem, thin stem and change in leaf lamina variants reappeared in \textit{in vitro} M$_2$ generation. The high frequency of long shoots and dwarf shoots could be observed on MS media incorporated with 0.05\% and 0.10 \% EMS which was 0.8, respectively. SA has also shown dwarf mutant but at very low frequency. All the three concentrations of EMS and 0.01 \% SA induced broad stem mutant. The highest frequency of broad stem mutant was recorded at 0.10 \% EMS which was 0.8±0.200 among the cultures. All concentrations of EMS showed thin stem variants in \textit{in vitro} M$_2$ generation. The 0.10 \% EMS has shown maximum induction of thin stem variants in \textit{in vitro} grown cultures. Low frequency of change in leaf lamina was recorded at
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0.10% and 0.15 % of EMS as well as 0.02% of SA. In the present investigation, EMS has been found to be the best mutagen to induce morphological variations in terms of long, dwarf shoots, broad and thin stem besides change in leaf lamina in *in vitro* propagation of *C. pictus*.

**Chlorophyll variants** (Table 18 and photo plate 15)

The leaf and nodal segments were excised from *in vitro* grown plantlets and subsequently inoculated on MS medium incorporated with mutagens SA and EMS. Cultures were kept in controlled aseptic conditions and observations were recorded. Results revealed that *in vitro* culture on 5th week exhibited morphological and chlorophyll variants as recorded in Table 18.

These *in vitro* grown cultures not only showed morphological variants but different types of chlorophyll variants as well. All these chlorophyll variants have been indicated in Table 18.

It is clear from Table 18 that different chlorophyll variants could get induced by mutagens SA and EMS incorporated in MS medium. Mutagens SA (0.01, 0.02 and 0.03%) and EMS (0.05, 0.10 and 0.15%) induced four types of chlorophyll variants namely *albino, xantha, chlorina and viridis*.

*Albino*: *In vitro* grown plantlets which showed whitish spots or entire leaf. They were observed after fourth week of inoculation. They failed to survive for a long time and after sixth week started shriveling.

*Xantha*: These plantlets have shown complete yellow to golden yellow coloured leaf spots or leaf. They were detected after third week of inoculation. In
these types of variants, the shoot height and survival percentage were found superior over *albino* and they survived till the 9 week.

**Chlorina:** *In vitro* grown plantlets which showed yellowish green colour and usually detected after fourth week of inoculation. They appeared quite similar morphologically to control. The survival percentage in them was superior over *albino* and *xantha* and they survived till the hardening of plantlets.

**Viridis:** *In vitro* grown plantlets, which exhibited dull green colour. The further *in vitro* growth changed them to normal green colour. They survived till the hardening stage of the plantlets.

In the present investigation, the explants, leaf and nodal segment were found potent to induce chlorophyll variants. Mutagens tried in present study (SA and EMS) succeeded in inducing chlorophyll variants. The highest mean frequency of chlorophyll variants could be was recorded at 0.10 % EMS, moderate frequency of chlorophyll variants was recorded at 0.05 and 0.10 % of EMS and 0.02 % of SA. The *xantha* variant was more frequent in case of mutagens SA and EMS, followed by *albino*. Mutagen EMS induced all the four types of chlorophyll variants however, SA was found less frequent to induce chlorophyll variants like *xantha, chlorina* and *albino* from the nodal segment explant.

Present study revealed that induced chlorophyll variants in *C. pictus* are possible by using SA and EMS mutagens under *in vitro* incorporating into media. It was found that EMS recorded best results to induce variability regarding chlorophyll variants using nodal segment as an explant. All the four types of
chlorophyll variants were induced in *in vitro* grown plantlets as shown in photo plate 15 and 16.

**C. PHYTOCHEMICAL ANALYSIS**

*C. pictus* plant has been recently introduced as an insulin plant, as its leaf and stem extracts have shown therapeutic effects in reducing sugar level. It is necessary to screen secondary metabolites to improve the plant by bioprospective potential as well as to screen the frequency of mutation induced. Considering such aspects, in present investigation the HRLC-MS technique was utilized to analyze the known as well as unknown secondary phytochemicals in *C. pictus*.

**High Resolution Liquid Chromatography - Mass Spectroscopy (HRLC- MS):**

The phytochemical composition of methanol extracts of leaf and stem part of *C. pictus* propagated by different methods such as *in vivo, in vitro*, mutagenic treatment were carried out in present investigation. All selected plant part extracts were analyzed using HRLC-MS technique by using devices of HiP sampler, Binary pump, column compound and Q-TOF. The gas temperature was programmed at 250\(^\circ\)C at gas flow 13/ minutes with 300\(^\circ\)C and 11/min of sheath gas temperature and sheath gas flow, respectively. About 5\(\mu\)l of methanol extract sample was injected into HRLC-MS instrument for analysis. HRLC-MS spectral analysis was carried out at Sophisticated Analytical Instrumentation Facility, Indian Institute of Technology (IIT) Powai, Bombay.

**HRLC-MS analysis of *Costus pictus*:**

*Analysis of in vivo leaf*
Experimental Results

Results of HRLC-MS of in vivo leaf samples of C. pictus revealed that there are 15 major compounds in methanol extract, which may contribute to the medicinal properties and qualities of plant. The major phytoconstituents present in methanol extract of in vivo grown leaf with retention time were 2-Amino-3-methyl-1-butanol (0.483), Tranexamic acid (0.646), L-2-Aminoadipic acid (0.977), 4-(2-hydroxypropoxy)-3,5-dimethyl-Phenol (5.756), 5-Methyl tetrahydropteroyltrii-L-glutamate (15.45), methyl-10-hydroperoxy-8E, 12Z,15Zoctadecatrienoate (16.18), Harderoporphyrin (17.512), 27-nor-5b-cholestane-3a,7a,12a,24,25-pentol (17.844), Avocadene Acetate (17.915), Ramiprilglucuronide (18.49), Rescinnamine (18.919), Trandolaprilglucuronide (20.499), all-trans-Nonaprenyl Diphosphate (22.249), 1-octadecanoyl-2(5Z,8Z,11Z,14Zeicosatetraenoyl)-sn-glycero-3-phosphate (22.774) and 1-octadecanoyl-2-hexadecanoyl-sn-glycerol (24.308), as listed in table 19.

Analysis of in vivo stem

Phytochemical investigation of in vivo grown stem revealed that, there are presence of 28 major constituents as confirmed by HRLC-MS technique. These constituents were Mebeverine metabolite (0.504), Zolazepam (1.785), 2-Furanpropionic acid tetrahydro -a- (1-naphthylmethyl) (3.5983), 4-bis(methylene)-Hexanedioic acid (4.655), Salicin (5.03), Dimethylcaffeic acid (6.168), Propanoic acid, 2-hydroxy-3-[(4-hydroxy-1-n napthalenyl)oxy] (8.642), Koparin 2'-Methyl Ether (8.643), Swietenine (9.006), Propanoic acid,2-hydroxy-3-[(4- hydroxy-1-naphthalenyl)oxy] (10.63), Trimethoprim (10.63), Linolenoyllysolecithin (12.03),
Harpagoside (12.162), GPEtn (10:0/11:0)[U] (12.779), Peruvoside (13.027), 1-Monopalmitin (13.028), Cetrimonium (13.337), Lactone of PGF-MUM (15.022), 3-Deacetyl Khivorin (17.085), Harderoporphyrin (17.5482), 7-nor-5b-cholestane a,7a,12a,24,25-pentol (17.801), Harderoporphyrin (18.054), Enkephaline, (D-Ala)2-Leu (18.527), Ramiprilglucuronide (18.528), Glycine,N-[(3a,5b,7a)-3- hydroxy-24-oxo-7-(sulfooxy)cholan-24-yl] (19.155), Antimycina (19.158), Trandolaprilglucuronide (20.269) and Docosanedioic acid (20.903), as listed in table 20.

**Analysis of in vitro grown leaves:**

To screen the changes in phytochemical constituents grown in in vitro leaf compared to in vivo, HRLC-MS technique was utilized. The analysis revealed constituents like Glucosamine (0.42), Triparanol (0.506), O-Acetylserine (0.609), Choline (0.641), Trolamine (0.648), Methylmn-Dimethylaniline-n-oxide(0.731), Norharman (4.168), Benzenemethanol,2-(2-aminoproxy)-3-methylmn (5.698), Linolenoyllysolecithin (12.04), Harpagoside (12.14), PheArgArg (12.741), 1-Monopalmitin (13.045), GPEtn (13.308), Lactone of PGF-MUM (14.842), Benzoquinone acetic acid (14.956), 10-hydroxy-hexadecan-1, 16-dioic acid (16.171), Harderoporphyrin (17.415), Enkephaline, (D-Ala)2-Leu (18.376), Ramiprilglucuronide (18.381), 3-DEOXO-3beta-Acetoxydeoxydihydrogedunin (18.986), 1,2-di-(9Z,12Zheptadecadienoyl)-Snglycerol (19.178), all-trans-Nonaprenyldiphosphate (19.191) and 1,2 di-(9Z,12Z,15Zoctadecatrienoyl)-3-O-Beta-Dgalactosyl-sn-glycerol (22.043) as major constituents as listed in table 21.
Experimental Results

Analysis of In vitro grown stem

The phytochemical investigation of in vitro grown stem was carried out by HRLC-MS at IIT Pawai, Mumbai. The methanol extract of in vitro stem revealed, 28 major constituents and they comprised 4-Trimethylammoniobutanal (0.481), Carnitine (0.523), dimethyl sulfoxide (0.545), O-Acetylsirine (0.612), 2-Amino-3-methyl-1-butanol (0.642), Trolamine (0.646), Arg Pro (0.647), Dimethylglycine (0.67), Tropicamide (3.476), Benzenemethanol, 2-(2-aminopropoxy)-3-methyl (5.689), Dihydrodeoxy-streptomycin (9.029), Swietenine (9.029), Linolenoyllyssolecithin (12.057), GPEtn(10:0/11:0)[U] (12.821), 1-hexadecanoylsn-glycerol (13.043), Peruvoside (13.049), Lactone of PGF-MUM (14.894), 10-hydroxy-hexadecan-1,16-dioic acid (16.152), C16 Sphinganine (17.264), Harderoporphyrin (17.38), N-(2- hydroxyethyl) palmitamide (17.632), 27-nor-5b-cholestane-3a,7a,12a,24,25-pentol (17.703), Enkephaline, (D-Ala)2-Leu (18.329), Ramiprilglucuronide (18.334), Antimycin A (18.929), 1,2-di-(9Z,12Z-heptadecadienoyl)-Snglycerol (19.161), all-trans-Nonaprenylidiphosphate (19.163) and 1,2-di-(9Z,12Z,15Zoctadecatrienoyl)-3-O-Beta-Dgalactosyl-sn-glycerol (21.967) as listed in table 22.

Analysis of in vitro grown mutagenic Leaf

To screen the in vitro mutation induction, the HRLC-MS analysis of mutagenic leaf was carried out. Results revealed Choline (0.445), O-Acetylsirine (0.606), 1-Aminocyclopropane-1-carboxylic acid (0.608), Arg Pro (0.643), L-2-Aminoacidipic acid (0.707), Norharman (4.146), Benzenemethanol, 2-(2-
aminopropoxy)-3-methyl (5.68), 4-2-hydroxypropoxy)-3,5-dimethyl-Phenol (5.68), D-Biotin (6.203), Swietenine (9.032), Dihydrodeoxy streptomycin (9.032), Phytosphingosine (11.128), Harpagoside (12.14), Cetrimonium (13.295), GPEtn(16:0/0:0) (13.327), Triamcinolone acetonide glucuronide (13.73), Lactone of PGF-MUM (14.814), Terephthalic acid (14.898), Lactone of PGF-MUM (14.93), Methyl8-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]Octanoate (15.731), Emedastine(16.147), SyringomethylReserpate (16.337), GPCho(O-18:2(9Z,12Z)/2:0) (16.589), C16 Sphinganine (17.252), Harderoporphyrin (17.358), 27-nor-5b-cholestan-3a,7a,12a,24,25-pentol (17.699), Harderoporphyrin (17.847), Enkephaline, (D-Ala)2-Leu (18.312), Ramiprilglucuronide (18.321), Garcinolic acid (18.398) and 3-deoxo-3beta-acetoxydeoxydihydrogedunin (18.913) as major constituents as listed in table 23.

**Analysis of in vitro grown mutagenic stem**

HRLC-MS analysis of mutagenic stem revealed Pyridostigmine (0.45), Choline (0.534), O-Acetylserine (0.598), Desmethylandansetron (0.602), Anabasamine (0.656), Trolamine (0.66), nn-Dimethylaniline-n-oxide (0.877), L-2-Aminoacidipic acid (0.962), Tropicamide (3.433), Norharman (4.083), D-Biotin (6.209), 8′,10′ dihydroxydihydroergotamine (9.039), Swietenine (9.039), Cetylpyridinium (11.871), Harpagoside (12.148), Cetrimonium (13.233), GPEtn (16:0/0:0) (13.335), Triamcinolone acetonide Glucuronide (13.73), Terephthalic acid (14.888), Syringomethyl Reserpate (16.331), C16 Sphinganine (17.232), 27-nor-5b-cholestan-3a, 7a, 12a, 24, 25-pentol (17.726), Harderoporphyrin (17.83),
Ramiprilglucuronide (18.302), 3-Deoxo-3beta-Acetoxydeoxydihydrogedunin (18.893) and Trandolaprilglucuronide (20.049) as major constituents as listed in table 24.

For release plant as herbal drug against diabetes further phytochemical analysis is needed. Plants should be studied through bioprospecting view and the standardization of dose may prove beneficial in nullifying the effect of palmitic acid. Here the objectives of alternative method for conservation and collection of preliminary phytochemical information have been attempted and fulfilled successfully.