Direct Organogenesis and Plantlet Establishment via Cotyledon Explants in Medicinally Important Herb *Silybum marianum* (L.)

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Abstract

Objectives: The species *Silybum marianum* (L.) (Asteraceae) commonly known as *Milk thistle* is medicinally valuable and due to over exploitation the species may become threatened. Hence, we have made an attempt to optimize the protocol for its direct regeneration through cotyledon explants using in vitro culture technology. Methods/Statistical Analysis: For in vitro multiple-shoot induction, cotyledon explants were excised from surface-sterilized seeds and cultured on MS Basal Medium (MBM) enriched with diverse conc. (10.0–20.0 µM) of BAP/KIN alone and also 10.0 µM BAP/KIN in combination with 1.25–5.0 µM NAA/IAA/IBA. The micro-shoots were cut out and cultured on 1/2 strength MBM enriched with diverse concentrations (5.0-20.0 µM) of NAA/IAA/IBA alone and also coupled with 1.25–5.0 µM BAP/KIN. Findings: In this study, superlative rate of shoot induction, maximal Number of Shoots Per Explant (NSPE) and longest shoot-length were achieved on MBM enriched with 10.0 µM BAP + 2.5 µM NAA. Superlative rate of root induction, mean number of roots per shoot and mean root length were documented on MBM supplemented with 15.0 µM NAA + 2.5 µM BAP. Application/Improvement: Being over exploited, the current technique can be operated for accelerated multiplication and conservation of the species. Further, this protocol can also be operated for transfer of novel genes for enhancement and modulation of bioactive molecules production in *S. marianum* – a medicinally valuable plant.

Keywords: Cotyledon, In vitro Regeneration, *Silybum Marianum*

1. Introduction

Seeds of *Silybum marianum* (L.) Gaertn (Asteraceae) contain silymarin, a mixture of flavonolignans (silychristin, silydianin, silybinin, isosilybinin) and taxifolin. Silymarin is commonly used to cure the liver damages and cirrhosis as well as certain cancers. Since, the species is medicinally valuable and due to over exploitation, this may become threatened. Hence it needs rapid multiplication and conservation through in vitro culture technology. Though it is economically important herb, the species *S. marianum* has been ignored in research programs for its improvement and conservation. So far, there are no reports on optimization of in vitro direct regeneration protocol using cotyledon explants in *S. marianum*. Hence we have optimized the protocol for its mass-scale production and conservation of the species by using in vitro the cotyledon explants.

2. Material and Methods

2.1 Plant Material

The seeds of were surface disinfected using drops of Tween-20 in 100 ml sterile distilled water for 20 min. Later sterilized with 4% (w/v) NaOCl for 5 min followed by washing in sterile distilled water three times for 5 min.
2.2 Culture Media and Culture Conditions
Cotyledon explants were excised from surface sterilized seeds and inoculated on MS Basal Medium (MBM) enriched with 3% (w/v) sucrose and various concentrations (10.0–20.0 µM) of BAP/KIN alone and also 10.0 µM BAP/KIN in combination with 1.25–5.0 µM NAA/IAA/IBA.

2.3 In vitro Rooting and Plantlet Establishment
The micro-shoots of *S. marianum* developed through cotyledon cultures were excised (3-4 cm length) and cultured on to 1/2 strength MBM enriched with diverse concentrations (5.0-20.0 µM) of NAA/IAA/IBA alone and also 15.0 µM NAA/IAA/IBA coupled with 1.25–5.0 µM BAP/KIN for in vitro rooting.

2.4 Acclimatization
The in vitro rooted plantlets of *S. marianum* were washed with sterilized-distilled water to clear away remains of agar. They were shifted to pots consisting of the soil mix-vermiculite:perlite:sand (1:1:1) and tenting with polythene bags to provide the RH (85-90 %) and kept in the culture room for acclimatization.

3. Results and Discussion
3.1 Multiple Shoots Induction via Cotyledon Explants
Cotyledon explants of *S. marianum* were inoculated on MSO (MS free PGRs), MBM enriched with diverse conc. (concentrations) (10-20 µM) of BAP/KIN and also 10 µM BAP/KIN coupled with various concentrations (1.25-5.0 µM) of IAA/IBA/NAA. The results are presented in Tables 1-2 and shown in Figures 1-6. Cotyledon explants did not respond on MSO medium. Multiple shoots induction was observed after one week of inoculation from cut ends of the cotyledon explants in all the conc. and comb. (combinations) of PGRs used. Superlative rate of shoot induction and as well as superlative NSPE were recorded at 10 µM BAP in comparison to all other conc. of BAP/KIN used individually. BAP alone was more effective than KIN when added in the medium Table 1. Similar results were also obtained in *Salacia Chinensis*. As the concentration increased fewer rate of shoot induction was recognized and also the NSPE was also found to be reduced in both cytokinins used. Similarity, it was also reported in *Salacia Chinensis*.

To know the synergistic effect, the cotyledon explants of *S. marianum* were also cultured on MBM fortified with 10 µM BAP/KIN in combination with 1.25-5.0 µM IAA/IBA/NAA Table 2. After addition of auxins in the medium along with the cytokinins, enhancement effect on multiple shoots induction was found in all the conc. of IAA/NA/NAA in comparison to BAP/KIN alone used Figures 1-7. 2.5 µM NAA + 10 µM BAP combination exhibited a synergistic effect where superlative rate of shoot induction (90%), maximum NSPE (24.18) and superlative average shoot-length (2.88 cm) were attained in *S. marianum* compared to all other concentrations and combinations of PGRs tested (Figures 1, 3, 5). Mean shoot-length was also documented with varied results based on the PGRs and their combinations used (Figures 2, 4, 6). All measured values viz. rate of shoot induction, average NSPE and average shoots-length were upgraded significantly (p ≤ 0.05) when set side by side with MBM supplemented with single cytokinin. Along these lines, it is indicated that the direct regeneration and the induction of number of multiple shoots/explant depends on the concentration and combination of PGRs present in the medium. These findings are in agreement with the results obtained in *Salacia Chinensis* and in *Salacia Chinensis*. Maximum shoot proliferation on MBM supplemented with NAA + BAP + Zeatin using cotyledon explants in cucumber has been reported.

In vitro regeneration through cotyledon was also reported in *Salacia Chinensis*. The correlation between mean NSPE and average shoot-length were significant and positive (0.810) at the 0.01 level (2-tailed) that presented in Figure 7.

Hence, for *Agrobacterium tumefaciens* intermediated genetic modification, the combination of 10.0 µM BAP + 2.5 µM NAA PGRs was used in *S. marianum*.
Figure 1. Effect of diverse conc. of NAA + BAP/KIN on multiple-shoots formation from cotyledon explants in *S. marianum*.

Figure 2. Effect of NAA + BAP/KIN on length of shoots (cm) induced from cotyledon explants in *S. marianum*.

Figure 3. Effect of IAA + BAP/KIN on multiple shoots induction from cotyledon explants in *S. marianum*.

Figure 4. Effect of IAA + BAP/KIN on length of shoots induced from cotyledon explants in *S. marianum*.

Figure 5. Effect of IBA + BAP/KIN on multiple shoots induction from cotyledon explants in *S. marianum*.

Figure 6. Effect of IBA + BAP/KIN on length of shoots induced from cotyledon explants in *S. marianum*. 
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### 3.2 In vitro Rooting and Plantlet Establishment

After developing the shoots from cotyledon explants, the in vitro root induction and acclimatization are important for formation of plants *in vitro*. The micro-shoots developed through cotyledon cultures of *S. marianum* were shifted onto 1/2 strength MSO, 1/2 strength MBM augmented with diverse conc. of NAA/IAA/IBA and also 15 µM NAA/IAA/IBA coupled with different conc. of BAP/KIN (1.25-5.0 µM). The results are presented in Tables 3 and 4 and shown in Figures 8-13.

Micro-shoots did not respond on 1/2 strength MSO medium without PGRs even after twenty days of incubation. In vitro root induction was observed after fifteen days of inoculation from the basal region of micro-shoots. Roots were developed in all the conc. of NAA/IAA/IBA alone used Table 3. Highest rate of root induction, mean number of roots/shoot as well as mean length of root were documented at 15 µM NAA in comparison to all other conc. of IAA/IBA used individually. At the time that the concentration of auxin elevated the rate of root induction was found to be upgraded. And also the mean NRPS and mean length of roots were also found to be more in *S. marianum*.

To know the effect of minor level of cytokinin together with upraised concentration of auxins on in vitro rooting, the micro-shoots were also shifted onto 1/2 strength MBM augmented with 15.0 µM IAA/NA/IBA in combination with 1.25 µM–5.0 µM BAP/KIN Table 4. Our observations

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**Figure 7.** Scatter graph and correlation comparison between NSPE and mean shoot-length induced from cotyledon explants in *S. marianum*. [The correlation between the mean NSPE and mean shoot-length was significant and positive (0.810) at the 0.01 level (2-tailed)].

**Table 1.** Effect of BAP/KIN on multiple-shoots induction from cotyledon explants in *S. marianum*

| Concentration of PGRs (µM) | Percentage of Shoot Induction | Mean NSPE (±SE) | Mean Shoot-Length (cm)(±SE) |
|---------------------------|------------------------------|----------------|-----------------------------|
| BAP KIN                   |                              |                |                             |
| 10                        | 52                           | 12.23±0.485ab  | 1.06±0.044a                |
| 15                        | 46                           | 11.26±0.545a   | 1.19±0.062a                |
| 20                        | 36                           | 8.56±0.687bc   | 0.79±0.054c                |
| 10                        | 22                           | 6.82±0.630abc  | 1.00±0.043a                |
| 15                        | 20                           | 6.30±0.335c    | 0.94±0.064bc               |
| 20                        | 16                           | 5.63±0.460abc  | 0.74±0.060d                |

Means within a column presented by the same letter are not different significantly according to DMRT (P ≤ 0.05).

**Table 2.** Effect of NAA/IAA/IBA + BAP/KIN on multiple-shoots induction from cotyledon explants in *S. marianum*

| Concentration of PGRs (µM) | Percentage of Shoot Induction | Mean NSPE (±SE) | Mean Shoot-Length (cm)(±SE) |
|---------------------------|------------------------------|----------------|-----------------------------|
| BAP KIN       NAA IAA IBA |                              |                |                             |
| 10.0          1.25          60          | 11.80±0.576e          | 1.49±0.271c    |
| 10.0          2.5           90          | 24.18±0.625a          | 2.88±0.480a    |
| 10.0          5.0           62          | 12.74±0.529d          | 1.18±0.308f    |
| 10.0          1.25          18          | 5.78±0.572d           | 0.78±0.233d    |
| 10.0          2.5           20          | 7.10±0.809h           | 0.85±0.259g    |
| 10.0          5.0           18          | 7.44±0.868hi          | 0.74±0.230h    |
| 10.0          1.25          60          | 10.77±0.531def        | 1.48±0.047c    |
| 10.0          2.5           80          | 19.75±0.594bi         | 2.41±0.055b    |
| 10.0          5.0           66          | 15.06±0.490c          | 1.52±0.050e    |
| 10.0          1.25          16          | 5.88±0.549d           | 0.75±0.068gh   |
| 10.0          2.5           20          | 10.20±0.929ef         | 0.94±0.097hf   |
| 10.0          5.0           18          | 6.33±0.645bi          | 0.69±0.084gh   |
| 10.0          1.25          44          | 9.32±0.540ef          | 0.94±0.056gf   |
| 10.0          2.5           58          | 12.55±0.512de         | 1.11±0.045d    |
| 10.0          5.0           50          | 10.68±0.427def        | 1.05±0.055hk   |
| 10.0          1.25          16          | 3.75±0.366d           | 0.54±0.046dh   |
| 10.0          2.5           24          | 8.58±0.712gh          | 0.96±0.051ef   |
| 10.0          5.0           20          | 8.40±0.653gh          | 0.82±0.053ef   |

Means within a column presented by the same letter are not different significantly according to DMRT (P ≤ 0.05).
implied that, all measured root induction values were expanded significantly ($p \leq 0.05$) when compared to 1/2 strength MBM fortified with single auxin. Ultimate rate of root induction (78%), superlative mean number of roots per shoot (11.77±0.355) and maximal mean root length (8.04±0.110 cm) were documented at 15.0 μM NAA coupled with 2.5 μM BAP compared to single auxin and other PGRs tested. Whereas IAA and IBA coupled with BAP were recorded lower impressive Figures 8-13 in inducing number of roots/micro-shoot. However, KIN + IAA/IBA/NAA combinations demonstrated diminished measured root induction values. Based on our surveillances, NAA had shown predominance over IAA and IBA in in vitro rooting. The best PGRs blend was found to be 2.5 μM BAP + 15.0 μM NAA for induction of profuse rhizogenesis in $S. marianum$. Using of high conc. of auxins individually or coupled with low conc. of cytokinins for in vitro rooting in several plant systems were also reported.

The correlation between the mean number of roots/explant and mean root length were significant and positive (0.791) at the 0.01 level (2-tailed) Figure 14.

Figure 8. Effect of diverse conc. of BAP/ KIN + NAA on in vitro root induction from cotyledon derived micro-shoots in $S. marianum$.

Figure 9. Effect of diverse conc. of BAP/ KIN + NAA on root-length in in vitro rooting of $S. marianum$.

Figure 10. Effect of diverse conc. of BAP/ KIN + IAA on in vitro root induction of micro-shoots developed from cotyledon explants in $S. marianum$.

Figure 11. Effect of diverse conc. of BAP/ KIN + IAA on root-length in in vitro rooting of $S. marianum$.

Figure 12. Effect of diverse conc. of BAP/ KIN + IBA on in vitro root induction of micro-shoots developed from cotyledon explants in $S. marianum$. 
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**Table 3.** Effect of NAA/IAA/IBA on in vitro root induction from cotyledon derived micro-shoots in *S. marianum*

| Concentration of PGRs (µM) | Percentage of Root Induction | Mean NRPS (±SE) | Mean Root-Length (cm) (±SE) |
|----------------------------|-----------------------------|-----------------|-----------------------------|
| NAA IAA IBA                |                             |                 |                             |
| 5                          | 20                          | 2.10±0.407d     | 3.00±0.265f                 |
| 10                         | 42                          | 2.38±0.305d     | 3.06±0.116f                 |
| 15                         | 64                          | 5.78±0.199a     | 6.80±0.118a                 |
| 1.25                       | 15.0                        | 5.90±0.199a     | 7.35±0.087b                 |
| 2.5                        | 15.0                        | 7.21±0.390d    | 8.04±0.110d                  |
| 5                          | 15.0                        | 8.00±0.408d    | 8.88±0.800c                  |
| 1.25                       | 15.0                        | 8.91±0.032c     | 7.35±0.629a                  |
| 2.5                        | 15.0                        | 3.33±0.284c    | 3.04±0.446d                  |
| 5                          | 15.0                        | 5.12±0.392h    | 4.05±0.642c                  |
| 1.25                       | 15.0                        | 4.14±0.294h    | 4.08±0.342c                  |
| 2.5                        | 15.0                        | 4.85±0.319h    | 5.77±0.144c                  |
| 5                          | 15.0                        | 8.78±0.363c    | 6.56±0.130d                  |
| 1.25                       | 15.0                        | 5.39±0.331f    | 5.89±0.104e                  |
| 2.5                        | 15.0                        | 4.18±0.226f    | 3.19±0.210e                  |
| 5                          | 15.0                        | 4.87±0.363f    | 4.27±0.135f                  |
| 1.25                       | 15.0                        | 3.92±0.265c    | 3.43±0.070d                  |

Means within a column presented by the same letter are not different significantly according to DMRT (P ≤ 0.05).

3.3 Plant Establishment

Plantlets with well grown leaves and developed roots were taken from the culture vessels and transferred into pots containing sterilized vermiculite:perlite:sand (1:1:1) and kept in culture room for acclimatization Figure 15h. After acclimatization, the in vitro grown plantlets were shifted into field and 55% of the plantlets were found to be survived. These in vitro regenerated plants were maintained in the field under shady conditions and they are morphologically similar to donor plant (Figure 15i).

**Table 4.** Effect of NAA/IAA/IBA + BAP/KIN on in vitro rooting from cotyledon derived micro-shoots in *S. marianum*

| Concentration of PGRs (µM) | Percentage of Root Induction | Mean NRPS (±SE) | Mean Root-Length (cm) (±SE) |
|----------------------------|-----------------------------|-----------------|-----------------------------|
| NAA IAA IBA                |                             |                 |                             |
| 1.25                       | 15.0                        | 5.00±0.335a     | 5.55±0.121a                 |
| 2.5                        | 15.0                        | 7.21±0.390d    | 5.72±0.113c                 |
| 5                          | 15.0                        | 6.08±0.270c    | 5.63±0.092c                 |
| 1.25                       | 15.0                        | 7.88±0.438c    | 5.88±0.800c                 |
| 2.5                        | 15.0                        | 8.91±0.032c    | 7.35±0.629a                 |
| 5                          | 15.0                        | 3.33±0.284c    | 3.04±0.446d                 |
| 1.25                       | 15.0                        | 5.12±0.392h    | 4.05±0.642c                 |
| 2.5                        | 15.0                        | 4.14±0.294h    | 4.08±0.342c                 |
| 5                          | 15.0                        | 4.85±0.319h    | 5.77±0.144c                 |
| 1.25                       | 15.0                        | 8.78±0.363c    | 6.56±0.130d                 |
| 2.5                        | 15.0                        | 5.39±0.331f    | 5.89±0.104e                 |
| 5                          | 15.0                        | 4.18±0.226f    | 3.19±0.210e                 |
| 1.25                       | 15.0                        | 4.87±0.363f    | 4.27±0.135f                 |
| 2.5                        | 15.0                        | 3.92±0.265c    | 3.43±0.070d                 |

Means within a column presented by the same letter are not different significantly according to DMRT (P ≤ 0.05).
4. Conclusion

Based on our results, it was revealed that the combination of NAA + BAP enhanced the development of higher NSPE as well as NRPS from cotyledon explants, whereas KIN had shown lower effectiveness for inducing NSPE and NRPS in *S. marianum*. Thus, the reproducible regeneration technique has been well-established in *S. marianum*. The same protocol can be used for rapid multiplication, conservation and *A. tumefaciens* intermediated genetic modification of this medicinally important herb.

5. References

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