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# Establishment of an efficient and reproducible *in vitro* protocol for callogenesis of *Silybum marianum*

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## Abstract

*Silybum marianum* (milk thistle) is belongs to astereaceae family and a wild herb in Pakistan. Silymarin is an isomeric mixture of flavonoids (silybin, silydianin, silibinin and silychristin) and a flavonolignans (taxifolin) extracted from seeds of milk thistle, and used against liver diseases. Due to problems in traditional cultivation and extraction of its bioactive compound (silymarin), different strategies have been employed in order to fulfil the scarce demand of *Silybum marianum*. *In vitro* micropropagation method has great potential to produce medicinal plants in large amount in less duration of time. So *in vitro* propagation protocol was established for this medicinal crop by using explants from 21 days old-seedlings induced *in vitro* from seeds. For efficient callogenesis, seeds were surface sterilized and cultured on hormone free $MS_0$ medium. After induction of seedlings, leaf, stem and hypocotyl were excised from the mother plants and used as explant for callus induction. Six different plant growth hormones (PGR) were checked for their callogenesis ability from three explants. It was obvious from the results that hypocotyl explant induce maximum callus induction frequency (100%) within minimum time i.e (11±2.87) days when cultured on MS+1mg/l 2,4-D+1mg/l IBA+1mg/l IAA (CIM-5) medium as compared to other medium. Hypocotyl explant was considered best explant for the highest production of callus as compared to leaf and stem explants of *S. marianum*.

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## Introduction

*Silybum marianum*, belongs to astereaceae family and commonly known as milk thistle due to presence of definite white (milky) veins in leaves. It has been used widely as a natural medicine for liver and biliary tract disorders (Shaarawy *et al.,* 2009). Silymarin is an isomeric mixture of flavonolignans and flavonoids such as silychristin, silydianin, silybin, and isosilybin, taxifolin (Wallace *et al.*, 2005; Engelberth*et al.*, 2008), extracted from milk thistle, is extensively used as hepatoprotective agent. Seeds and fruits are the abundant source of silymarin as compared to other part of the plant (Saller *et al.,* 2001). The hepatoprotective effect of silymarin is mediated through different mechanisms, including antioxidant and free radical scavenging activities (Shaker *et al.*, 2010), and hepatocellular membrane stabilization (Basiglio *et al.*, 2009) and cell permeability regulation, anti-inflammatory activities (Gupta *et al.*, 2000), liver regeneration stimulation and inhibition of collagen fibers deposition. In addition to treating liver disorders, silymarin has beneficial property on a wide variety of other disorders, such as anticancer effect (Öztürk *et al.*, 2015), prevention of hemolysis of red blood cells (Zou *et al.*, 2001)and renal protection (Soto *et al.*, 2010; Rafieian-Kopaie and Nasri, 2012), hypolycemic activity and prevention of insulin resistance (Huseini *et al.*, 2006).

At present, the increasing demand for silymarin is endangering the *S. marianum* populations (Ahmad *et al.*, 2008). Moreover, the efficiency of seed germination and seedling growth, which highly depends on various biological and environmental factors, is very low (Shinwari *et al.*, 2014). Besides, manual handling of the plants is very difficult because leaves and flowers are spiny and, as the plant is cultivated in rows, using harvesting machine causes damage and reduces the crop yield (Hammouda *et al.*, 1993).

*In vitro* propagation, is an alternative (unconventional) method for the production of active ingredients of medicinal plants. Enough plant material and secondary metabolites availability round the year are the main advantages of *in vitro* micropropagation of plants. Medicinal plants are the incredible source for the development of drugs, in this regard different biotechnological methods are potential source for the higher accumulation of secondary metabolites. Depending upon the capability of the tissue culture system thousand or even millions of plants can be produced from a single plant (Vanisree *et al.,* 2004; Nikolova and Ivancheva, 2005; Elfahmi, 2006; Gopi and Vatsala, 2006).

To the best of our knowledge in Pakistan, there are no studies related to the micropropagation from seeds of this plant. Then there is great need of increasing the production of this hepatoprotective herb, and used these products for the *in vitro* studies of the secondary metabolites from therapeutically important medicinal plants.

## Materials and methods

The main purpose of present research was to establish an efficient and reproducible in vitro protocol for callogenesis of *Silybum marianum*. Six different media were used for induction on different callus induction medium from three different explants. Seeds of milk thistle were sampled from Postgraduate Agriculture Research Station (PARS), University of Agriculture Faisalabad.Firstly, seeds of milk thistle (*Silybum marianum*) were rinsed with autoclaved distilled water and then seeds were dispensed in flask for disinfection with 70% ethanol (v/v) for 10 minutes for the removal of contaminants and dust from surface. Followed by sterilization with 50% commercial bleach in addition to tween-20 and then placed on shaker at 120 rpm for 15 minutes and finally seeds were washed 4-5 times with autoclaved distilled water to remove bleach and tween-20 completely as described by Eari *et al.* (2016).

*Germination of seeds*

For the germination of seeds, procedure described by Pourjabar*et al.* (2012) was followed. Murashige and Skoog basal medium ($MS_0$) without fortification of plant growth regulators was prepared. The pH was

adjusted to 5.8-5.84 with 0.1M NaOH or HCl. The media was then dispensed in glass bottles for autoclave. After that seeds were cultured on sterilized $MS_0$ basal medium under aseptic conditions in the laminar airflow for the germination of seeds. These test tubes were wrapped with autoclaved polypropylene sheets and incubated under controlled conditions i.e16 hours light *and* 8 hours dark photoperiod at 25°C ± 2.0 in growth room. The *in vitro* generated seedlings were then used as explants source.

*Stock Solution preparation*

Stock solution of plant growth regulators (2,4 dicholoro-phenoxy acetic acid, α-Naphthalene acetic acid, Indole-3-butyric acid, 6-benzylamino-purine, Gibberellic acid, Kinetin and Thidiazuron ) were prepared by using the protocol prescribed by Davey and Anthony, (2010) in ethanol or NaOH and final volume was adjusted by using autoclaved distilled water.

*Preparation callus induction medium (CIM)*

Callus induction was optimized at different media to get higher amount of callus at minimum interval of time. Total six callus induction media were used and exploited against the three explants (stem, leaf and hypocotyl).

*Preparation of Explants for Callus Induction*

From 20-day old seedling, explants were excised and prepared for callus induction by the procedure documented by John and Koperuncholan, (2012).Leaf explants were taken fromthree week old seedlings of milk thistle and leaf was detached from stem with sharp sterilized blade and forceps and used as explants. Stem explant was prepared by excising approximately2-3mm size from 21-day old seedling and used as explant for callogenesis. Upper short portion of hypocotyl from root was cut with blades and used as explant. After the preparation of callus induction medium, prepared explants were cultured on all six medium and response was recorded.

*Growth Parameters of Callus induction*

Growth parameters were calculated for each explant and medium and data was recorded as described by Rady*et al.* (2013) on the following parameters having ten replication per treatment. The days required to initiate callus by explants cultured on different callus induction media were recorded in this experiment.

*Percent callus induction*

Percent callus induction was calculated after four to six weak of callus initiation by using following method:

$$\text{Callus induction (\%)} = \frac{\text{Number of explants inducing callus}}{\text{Total Number of cultured explants}} \times 100$$

*Callus Morphology*

Callus morphology data including callus texture and color was recorded for each subculture by visual observations. Data were recorded from all the explants used in this medium by the method documented by Arif*et al.* (2014).

**Results**

*Days to callus induction*

Three explants were exploited on CIM-1 (MS + 0.25 mg/L 2,4-D + 0.25 mg/L Kin), and days to callus induction were estimated.

**Table 1.** Morphology of callus at callus induction medium and explant.

| Media | Hypocotyl | Leaf | Stem |
|---|---|---|---|
| CIM-1 | Whitish yellow, less proliferated | Brownish yellow, compact | Whitish yellow, hard |
| CIM-2 | Pale yellow, less proliferated | Brownish yellow, friable | Yellow, friable, |
| CIM-3 | Whitish yellow, less proliferated | Brownish yellow, less proliferated | Whitish yellow, non-Friable |
| CIM-4 | Whitish yellow, friable, | Brownish yellow, friable | whitish yellow, less proliferated |
| CIM-5 | Whitish yellow, granular, highly proliferated, soft | Brownish yellow, less proliferated | Greenish yellow, less proliferated |
| CIM-6 | Greenish yellow, hard, friable, compact, well proliferated | Greenish yellow, Granular, less proliferated | whitish yellow, less proliferated |

It was observed that minimum days recorded were same in stem (39 ± 4.87), leaf (39 ± 5.13) and much higher in hypocotyl (43 ± 4.38) explant. Statistically results showed that no significant difference in days to callogenesis in different explants as shown in Fig. 1.

CIM-2 was also checked for its potential to initiate callus from stem, leaf and hypocotyl explants. It was revealed from the results that 38 ± 5.32, 40 ± 6.03 and 42 ± 2.85 days were required to initiate callus from stem, leaf and hypocotyl respectively.

It was obvious through results that there was no significant effect of CIM-2 on days required for callogenesis (Fig.1). Callus induction media-3 was employed on hypocotyl, stem and leaf explants, and days to callus initiation were recorded. The callogenesis potential of hypocotyl, leaf and stem explants in terms of days to callus induction was 39 ± 5.87, 42 ± 5.23 and 44 ± 5.27 respectively as shown in Figure 1.

Culturing of explants on CIM-4 containing MS medium supplemented with 4.5 mg/L 2,4-D, revealed its non-significant effect in callogenesis, explant was documented. Maximum days were taken by hypocotyl (44 ± 5.40), followed by leaf (43 ± 5.74) and stem (42 ± 4.65), as presented in Fig. 1. CIM-5 induced callus from hypocotyl within minimum time period i.e, (11 ± 2.87 days) from cultured date as compared to stem and leaf which initiated callus after 43 ± 4.42 and 43 ± 7.46 days respectively. Callus initiation and proliferation from hypocotyl explant presented in Fig. 2.

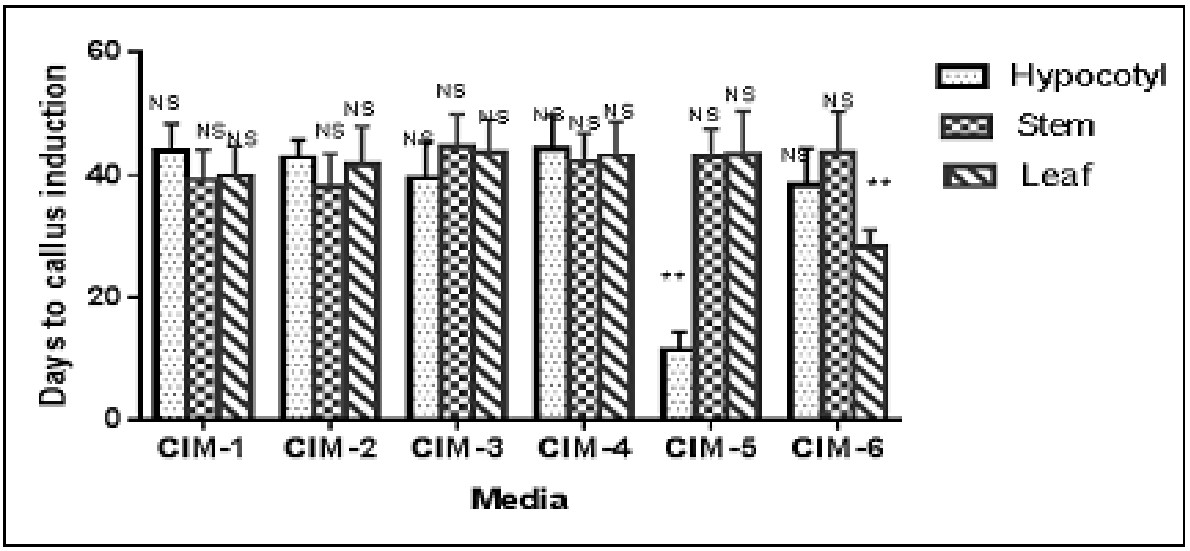


**Fig. 1.** Days to callus induction on different medium and explants.

Significant difference between explant callogenesis ability was found, as hypocotyl took minimum days (11 ± 2.87) for callus induction as compared to leaf and stem explant (Fig. 1). It was also observed that leaves and stem were non-significant towards callogenesis on CIM-5. When explants were cultured on CIM-6, the results revealed that no significant difference was recorded in days required to callus initiation from hypocotyl (38 ± 5.92) and stem (43 ± 6.85). On the other hand significant difference in callus induction from leaf explant as that took minimum time (28 ± 2.50) for callus induction as represented in the Figure 1. Initiation and proliferation of callus from leaf explant presented in Fig.2. Upto this level by considering days to callus induction, four medium (CIM-1,2,3,4) showed no difference in callogenesis ability, but CIM-5 and CIM-6 were potent enough to induce callus from hypocotyl and leaf explant respectively as shown in Fig. 1.

*Callus Induction Frequency*

Callus induction frequency was calculated for all six media and three explants. Highest frequency of callus production was observed in hypocotyl, followed by

leaf and stem explant. Highest callus induction frequency (100%) was recorded in hypocotyl when the MS medium fortified with combination of 1mg/L 2,4-D + 1mg/L IBA + 1mg/L IAA was used **(**Fig. 3**)**. The minimum callus induction frequency (15%) was also observed in hypocotyl on CIM-3.

Callogenesis frequency (95%) of leaf was lower but significantly higher when cultured on CIM-6 as shown in Fig. 3.

Consequently, it was revealed from the results that hypocotyl and leaf explants showed maximum callus induction frequency and required minimum days for callus induction. Results were correlated with the finding of Munaf *et al.* (2009) who found that hypocotyl was the most convenient explant for callus formation. Whereas, Eari *et al.* (2017) found maximum callus induction from leaf explants on MS medium supplemented with 0.5 and 1 mg/L 2,4-D and Kin. In the present study the leaf explant showed 95% callus induction frequency when cultured on MS + 1mg/L 2,4-D + 0.5 mg/L BAP (CIM-6), Eari *et al.* (2017) endorsed the finding that leaf explant is the most responsive to induce callus formation. Yu-weilw *et al.* (2017) also observed that MS fortified with 1.5 mg/L BAP and 2.0 mg/L NAA induced highest frequency (98.9%) of callus induction from immature leaf pieces. Abbasi *et al.* (2010) also developed callus from leaf explants on media supplemented with different growth regulators, maximum percent callus induction frequency was recorded on MS medium supplemented with 5.0 mg/L 6-benzyladenine (BA) after 20 days.

*Callus Morphology*

In addition to other callus induction parameters, the morphology of the produced callus was also checked at all media and explant. Overall, friable and yellow in color callus of milk thistle was observed (Table 1).

Highest callus induction was observed in hypocotyl explant on CIM-5 (MS medium supplemented with 1mg/L 2,4-D, IBA+1mg/L IAA), and the morphology of callus was soft, whitish yellow, granular and highly proliferated. Whereas, maximum callus induction in leaf was found on MS medium supplemented with 1mg/L 2,4-D+0.5mg/L BAP (CIM-6) and nature of the callus was greenish yellow, hard, compact, friable and well proliferated. Most of the produced callus were whitish yellow and friable (Fig. 2).

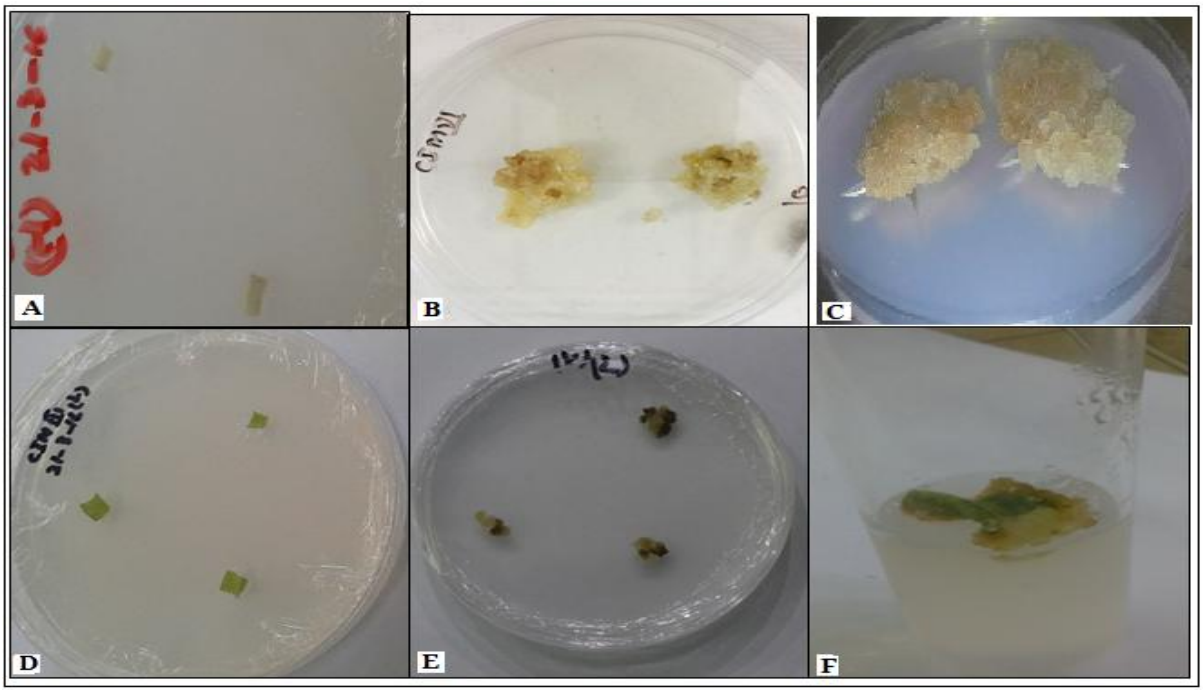


**Fig. 2.** Potential of CIM-5 and 6 on callogenesis ability of Hypocotyl and leaf explant: A: Hypocotyl Explant, B: Callus Initiation, C: Callus Proliferation, D: Leaf Explant, E: Callus Initiation, F: Callus Proliferation.

John and Koperuncholan, (2012) also induced callus from leaf and hypocotyl explant and found that yellow friable calli were produced from both explant. Eari *et al.* (2013) observed that when leaf was used as explant for callogenesis light green friable callus was produced when cultured on MS medium supplemented 0.5 mg/L 2,4-D and 0.1 mg/L Kin.

After calculation of callus induction parameters, it was concluded that CIM-5 (MS medium supplemented with 1mg/L 2,4-D+1mg/L IBA+1mg/L IAA) was optimal medium for callus induction from hypocotyl explant and MS medium supplemented with 1mg/L 2,4-D+0.5mg/L BAP (CIM-6) was the best for leaf explant. Stem was not responsive to all employed medium for callus induction (Fig. 3).

It was observed from the above mentioned results that there was a significant effect of explant on the callus induction parameters. Leaf and hypocotyl explants showed significant effects on callus induction parameters as compared to stem. The order of explants for callogenesis ability was hypocotyl>leaf>stem. Hypocotyl is a convenient explant for maximum callus induction (Liu and Cai, 1990; Sanchez-Sampedro *et al.*, 2005). Hypocotyl was considered as optimal explant for callogenesis (Munaf *et al.*, 2009). Moreover, leaf explant also showed significant callogenesis ability. Mean comparison of the effect of explant type revealed that leaf explant is the most responsive to produce higher fresh weight (Eari *et al.*, 2013). All explants were exposed to different culture medium to observe its effect on callus induction. CIM-5 was found best for callus induction from hypocotyl explant. Likewise, the callus from hypocotyl explant of *S. marianum* may also be induced successfully, if cultured on MS medium supplemented with 1mg/L 2,4-D and 0.5 mg/L BAP (Sanchez-Sampedro *et al.*, 2005). Same medium was examined for hypocotyl, leaf and stem explant for callus induction. Maximum callus induction frequency (95%) was observed from leaf explant as presented in Fig. 3.

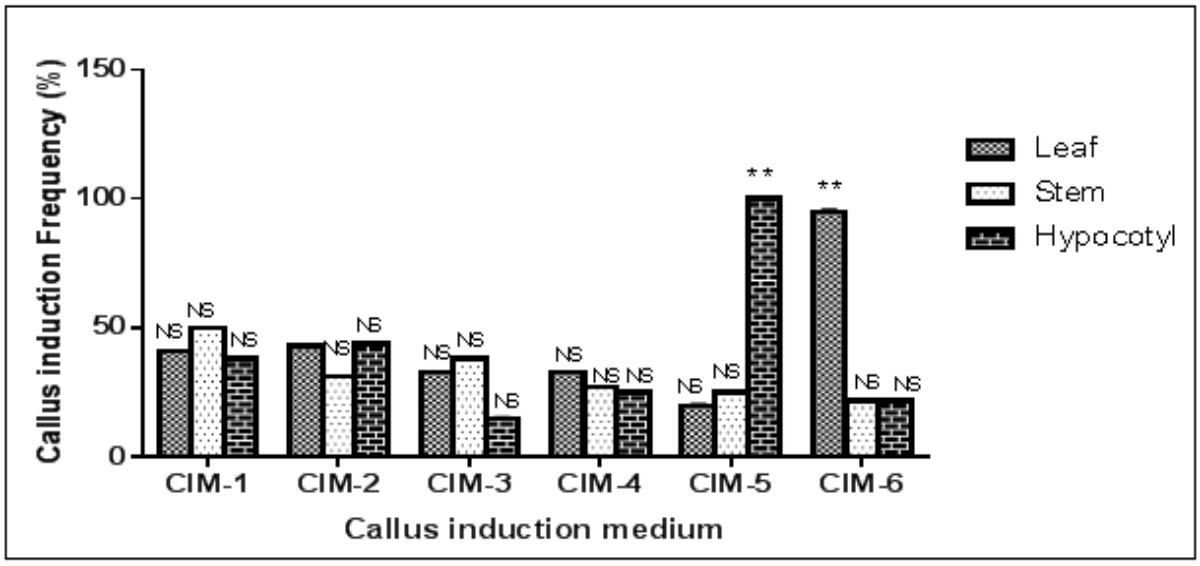


**Fig. 3.** Callus induction frequency on different medium and explants.

Callus could be induced from hypocotyl, leaf, petiole and stem explants from *in vitro* grown plantlets and frequency of callogenesis depends on composition of cultured medium (Bakheet *et al.*, 2013). Medium and explant show synergistic effect on callogenesis ability. It was obvious from the result (Fig. 1,3) that explant, hypocotyl, induces maximum callus when cultured on CIM-5 as compared to all studied medium. It was, therefore, concluded that hypocotyl considered as optimal explant for callogenesis on CIM-5. Previously, hypocotyl was also used as explant to induce callus on MS supplemented with 0.8 mg/L, 0.5 mg/L and 200 mg/L of NAA, BA and casein hydrolysate respectively (Liu and Cai 1990). In present study, leaf explant also

showed better response in terms of callus induction frequency (95%) on CIM-6 as displayed in Figure 3. In another study leaf explant gave highest frequency of callus induction MS medium supplemented with 5 mg/L kin and 0.5 mg/L IAA (Bakheet *et al.*, 2013).

Furthermore, cotyledons, (embryonic leaf), explant of *S. marianum* cultured on B5 medium (MS + 0.05 mg/L BA and 0.5mg/L 2,4- dicholorophenoxyacetic acid) induced highest callus induction frequency (98.9%) (Cimino *et al.* 2006).

## Conclusion

The findings of the present study suggested that *in vitro* micropropagation method has great potential for large scale production of *Silybum marianum*, and hypocotyl considered most suitable explant for callus production in large amount in minimum time duration through *in vitro* culture. This pilot study will lead to mass production of secondary metabolites of *Silybum marianum*.

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