LETTER TO THE EDITOR

EFFECT OF EXPLANTS AND GROWTH HORMONES ON DIRECT REGENERATION OF *SILYBUM MARIANUM IN VITRO*

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To the Editor,

Silybum marianum, also known as milk thistle, has been used as a hepatoprotective herb against liver ailments for 2000 years. Silymarin is a biomarker of *S. marianum*, a mixture of seven flavonolignans and flavonoid (1). The hepatoprotective activity of milk thistle is well documented among all medicinal plants (2).

Herbal therapy has attracted great attention as an alternative approach to cure liver disease. High quantities of Silymarin as a potential medicinal bioactive against liver disease are therefore needed (3). There are different traditional constraints, including spiny leaves and flowers, and the plant is cultivated in rows and the use of combine harvesters reduces production to 40% (4). Plant tissue culture is considered to be one of the best strategies for the hyper-production of valuable secondary metabolites on a large scale. The sufficient availability of plant material and secondary metabolites throughout the year is the main advantage of *in vitro* micropropagation of plants.

MATERIALS AND METHODS

Direct organogenesis is the method of tissue culture in which the plant is derived from any part of the explant using different growth regulators. Different *in vitro* generated explants, including leaves and shoots, have been evaluated for their ability to induce direct organogenesis and subsequent proliferation. The regeneration medium was fortified with different combinations of BAP, Kin, NAA and GA3 and the percentage of direct organogenesis was calculated after five weeks of cultivation.

Regeneration medium

Regeneration media were prepared in Murashige and Skoog (MS) basal medium fortified with growth hormones including BAP, Kin, NAA (1mg/L each) as shown in Table I. Direct regeneration was optimized in different media to regenerate the entire plant. Six different direct induction media (DRIM) were used to check the regeneration capacity of leaf and shoot explants (Table I).

During direct regeneration, leaves and roots were induced from the same induction media. Shoots and leaves were cultivated on a direct regeneration induction medium and shooting days, number of shoots and regeneration frequency were recorded. Rooted plantlets were exposed to bavistin solution (1%) to prevent contamination prior to transfer to soil-containing plastic pots. They were acclimatized under controlled temperature (25±2°C)

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and covered with transparent plastic bags to maintain humidity. The survival rate (percentage) of the plantlets was measured (5).

Statistical analysis

In the tissue culture experiment, 10 replicates per treatment were used and the data obtained was

Table I. *List of medium used for direct regeneration.*

Media Type	Media composition	Explants	Replicate
DRIM-1	MS+0.5mg/L BAP+1.5mg/L GA3+1mg/L TDZ	Leaf, Shoots	**10**
DRIM-2	MS+1mg/L GA3	Leaf, Shoots	**10**
DRIM-3	MS+1mg/L NAA+2mg/L GA3	Leaf, Shoots	**10**
DRIM-4	MS+TDZ (11µM)	Leaf, Shoots	**10**
DRIM-5	MS+1mg/L BAP+2mg/L NAA	Leaf, Shoots	**10**
DRIM-6	MS+1mg/LNAA	Leaf, Shoots	**10**

DRIM: direct regeneration induction media; BAP: 6-Benzylaminopurine; GA3: Gibberellic acid; TDZ: Thidiazuron; NAA: Naphthaleneacetic acid

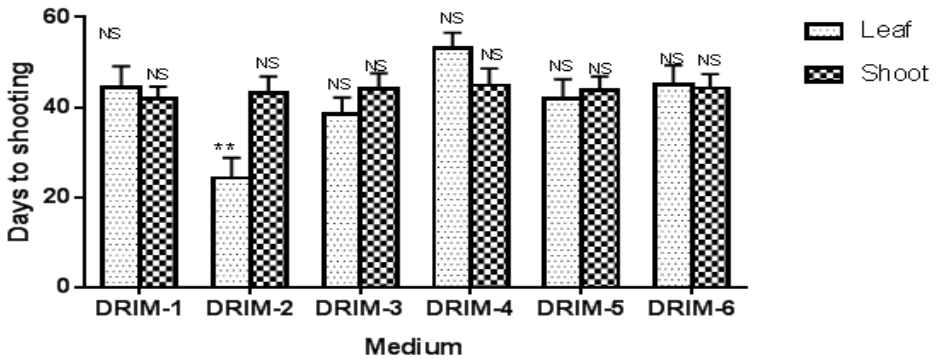

Fig. 1. *Days to shooting from leaf and shoot explant on different media. ** significant difference between the different media; [NS] non-significance difference between the different media. Values are presented as mean±SEM (n=10).*

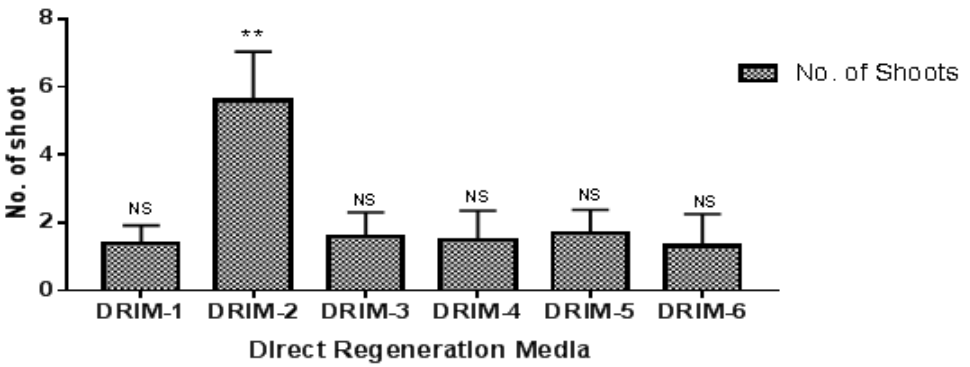

Fig. 2. *Number of shoots from leaf explant on different media. ** significant difference between the different media; [NS]non-significance difference between the different media. Values are presented as mean±SEM (n=10).*

statistically analyzed using Graphpad Prism 7.1 software. ANOVA and Turkey Multiple Comparison Test were used to compare different treatments for their regeneration capacity.

RESULTS

The direct regenerative potential of the leaf and shoot explant was examined in six different regenerative media. Leaf explant showed effective initiation of shoot within a minimum period of time i.e. 24±4.47 days after inoculation on MS medium fortified with 1mg/L GA3 (DRIM-2) as displayed in Fig. 1. The yellowish leaf blades were separated and discarded to minimize the movement of the leaf explants from the medium and successive elongation of the shoot buds. These regenerants were then subcultured on the same MS medium supplemented by 1mg/L GA3 and produced a maximum number of shoots/explants i.e., 6.5±1.43 compared to other media (Fig. 2). Improved direct organogenesis response was found in shoots grown on DRIM-1 medium containing a combination of 0.5mg/L BAP, 1.5mg/L GA3 and 1mg/L TDZ which produced shoots after 42±2.62 days (Fig. 1). The maximum regeneration frequency (93.33±2.367%) was achieved by the leaf explant on DRIM-2 (MS complemented with 1mg/L GA3) compared to the other media. While the MS medium supplemented by 0.5mg/L BAP+1.5mg/L GA3 + 1mg/L TDZ (DRIM-1) induced the lowest regeneration frequency (20±0.21%) from leaf explant (Fig. 3). The results showed that DRIM-2 was the best medium for direct somatic embryogenesis compared to the other media as shown in Fig. 2.

Finally, the direct regeneration plantlets were moved to sterilized soil and placed under controlled conditions in the growth room for two weeks prior to transfer to pots. A total of 25 *in vitro* rooted plantlets were planted in plastic pots, of which 20 (80%) survived. In addition, no morphological variations were examined as compared to the parent plant.

Leaf and shoot explants were checked for their ability to regenerate directly into the entire plant. It was shown from the results that the leaf has more potential to regenerate the whole plant compared to the shoot explant. Direct regeneration induced the shoots and roots simultaneously, but there was a difference in the days of initiation and the frequency of regeneration, as shown in Fig. 1, 2, 3. The ability to regenerate directly was also influenced by the medium composition. The MS medium supplemented by 1mg/mL GA3 (DRIM-2) showed the highest regeneration capacity compared to the other media.

DISCUSSION

There are two modes of plant regeneration, indirect (with) or direct (without), based on the participation of callus. Direct organogenesis appears to be superior to indirect organogenesis, as indirect regeneration from

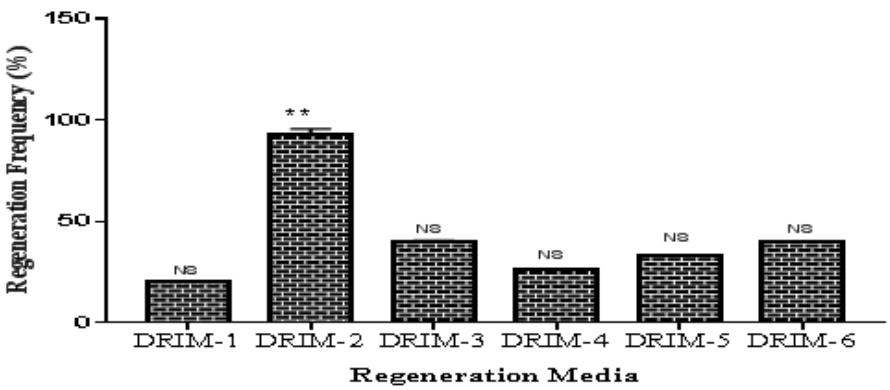

Fig. 3. *Regeneration frequency of leaf explant at direct regeneration induction medium. ** significant difference between the different media; [NS] non-significant difference between the different media. Values are presented as mean±SEM (n=10).*

callus may differ from parent plants. The ability to regenerate directly was also influenced by the medium composition. The MS medium supplemented by 1mg/mL GA3 (DRIM-2) showed the highest regeneration capacity compared to the other media. The maximum number of shoots was also observed on MS+1mg/mL GA3. While the minimum regeneration capacity in terms of days to organogenesis, number of shoots and regeneration frequency was observed on MS+1mg/LNAA+2mg/L GA3 medium as shown in Figs. 1, 2, 3. In the present study, GA3 may have been responsible for effective direct organogenesis from leaf explants. It has also been reported that leaf explant has the maximum efficacy of direct organogenesis compared to another explant (6). In addition, Arif et al. 2014 (7) also reported that leaf explant was more effective for direct regeneration compared to nodal and internodal explants.

The optimal medium was reported to be MS+3.0mg/L GA3 and 1.0mg/L NAA for direct organogenesis of milk thistle from leaf explants (5). However, BAP+NAA could be more suitable for direct organogenesis as 60 percent direct organogenesis frequency was observed on the MS medium supplemented by 1mg/L BAP and 2mg/L NAA (8, 9). However, in the current study, leaf explant was less responsive to direct regeneration when NAA was used in combination with GA3, TDZ, and BAP. In the present study, 20 (80%) out of 25 plantlets were successfully acclimatized with no morphological changes compared to the parent plants. Al-Hawamdeh et al. (10) also discussed the successful acclimatization of rooted milk thistle transplants with almost 70% survival; 22 (74%) of the 30 plantlets of Abbasi et al., survived (11). According to Yu-weilv et al. (5), 46 of the 50 milk thistle plantlets *in vitro* survived. El Sheriff et al. (12) also recorded a 100 percent survival rate for *in vitro* plantlets.

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