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THIDIAZURON ENHANCED REGENERATION AND SILYMARIN CONTENT IN *SILYBUM MARIANUM* L.

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Abstract

Silybum marianum, of family Asteraceae is renowned for production of biologically important silymarin, which has shown multi-dimensional medicinal properties. It has a high protective role against jaundice and hepatitis C worldwide. We hereby established a feasible and efficient method for indirect regeneration of *S. marianum* for production of consistent plantlets. Calli were induced from leaf explants of seed-derived plantlets on Murashige and Skoog (MS) medium supplemented with several concentrations of different plant growth regulators (PGRs). Highest callogenic response (89%) was recorded for 4.4µM Thidiazuron (TDZ) in combination with 6.6µM Kinetin (Kn). Subsequent sub-culturing of callus after 4 weeks of culture, on medium with similar compositions of PGRs induced shoot organogenesis. Highest shoot induction frequency (86%) with maximum mean multiple shoots (26 shoots per explant) were recorded for 11µM TDZ after 4 weeks of transfer. Longest shoots (4.1 cm) were recorded for MS medium augmented with 6.6µM TDZ and 4.4µM α-naphthalene acetic acid (NAA). Furthermore, rooted plantlets were developed on MS medium containing different concentrations of indole acetic acid (IAA). Silymarin was determined by High performance liquid chromatography (HPLC) and 8.47 mg/g DW silymarin was detected in the regenerated plantlets. This study contributes to a better understanding of the different mechanisms involved in morphogenesis and production of biologically active principle in *Silybum marianum*.

Introduction

Silybum marianum L. Gaertn., belongs to family Asteraceae and is commonly known as Milk thistle, it is native to the Mediterranean basin and is now widespread throughout the world. It is cultivated as a medicinal plant (Khan *et al*., 2009) and has been used from ancient times (Kren & Walterova 2005). It has an isomeric mixture of multiple flavonolignans called silymarin that is considered as the most potent antioxidant (Khan *et al*., 2013). Approximately 8 to 33% of patients with hepatic disorders consume this plant frequently as herbal remedy worldwide (Polyak *et al*., 2007). However, there are some issues with *Silybum* products, as existing high variability in the end products has resulted inconsistent results regarding its efficacy in various clinical trials (Lee & Liu, 2003; Haban *et al*., 2009). Therefore, control, characterization and standardization of silymarin appear to be a necessary mandate for formulation of end products with consistent efficacy (Aruoma, 2003). Consistent production of phytochemicals can be achieved via *In vitro* clonal propagation **(**Parveen & Shahzad, 2010**)**. The increasing world's demand for silymarin (18-20 tons per year) is endangering the sparse populations of this species, still this economically important herb has been ignored in research programs for its improvement and cultivation (Ahmad *et al*., 2008). Plant *In vitro* technology is capable of producing large number of aseptic, genetically similar, and chemically consistent plants in a short period of time and limited space for the production of high quality medicines (Victorio *et al*., 2012).

Thidiazuron (N-phenyl-N-1,2,3-thidiazol-5-yl urea) has proven to be highly effective bio-regulator of plant morphogenesis. It is reported to be much more effective in organogenesis via callogenesis in many plant species than the commonly employed conventional cytokinins (Wannakrairoj & Tefera, 2012; Hussain *et al*., 2013). Initially TDZ was classified as cytokinin due to its cytokinin like responses. However, later on research showed that TDZ is capable of fulfilling both the cytokinin and auxin requirement of various regenerative responses in many plant species (Arshad *et al*., 2012). Furthermore, TDZ application has a profound effect on enhancing the levels of secondary metabolites in the *In vitro* cultures of many medicinally important plant species (Liu *et al*., 2007).

The main objective of present study was to exploit TDZ for establishment of efficient regeneration protocol for *S. marianum* and to evaluate its impact on accumulation of silymarin in regenerated tissues and plantlets by HPLC.

Materials and Methods

Viable seeds of *Silybum marianum* were collected from main campus of Quaid-i-Azam University, Pakistan. *In vitro* seed germination was achieved by the feasible protocol reported by Khan *et al*., (2013). Leaf explants (1.5 cm^2) were excised from *In vitro* germinated 28-days old seedlings and were placed onto Murashige and Skoog basal medium (MS, 1962; Phytotechnology Labs, USA) containing 3% sucrose and 0.8% (w/v) agar (Phytotechnology Labs, USA) in 150 ml conical flask supplemented with various PGRs like Thidiazuron (TDZ), Kinetin (Kn) and α-napthalene acetic acid (NAA). Different concentrations (1.0, 2.2, 4.4, 6.6, 8.8, 11, 13.2 and 15.4µM) of TDZ or (1.0. 2.2, 4.4, 6.6 and 8.8 µM) of Kn or NAA either alone or in combinations were tested. The pH (Eutech Instruments pH 510, Singapore) of media was adjusted to 5.8 before autoclaving (121°C, 20 min, Systec VX 100, Germany). MS basal medium (MS0) without any plant growth regulator (PGR) was used as control for different phases of regeneration.

After 4 weeks, number of responding explants (% callus induction) was recorded. Callus was refreshed by sub-culturing to MS medium with similar composition of PGRs for further growth and multiplication.

Data on % shoot induction response; number of shoots per explant and mean shoot length (cm) was collected after 4 weeks of callus sub-culturing. Elongated shoots were transferred to rooting medium for root induction. Rooted plantlets were removed from flasks, washed with double distilled water and transferred to pots for acclimatization. For fresh weight (FW) determination, the plant samples were gently pressed on filter paper to remove excess water and weighed. Subsequently, the plant materials were dried in oven at 35°C for 24 h and dry weight (DW) was recorded.

Silymarin assessment by HPLC: For silymarin content determination, different samples (calli, regenerated shoots, regenerated plantlets, and growth room potted plantlets) were subjected to High Performance Liquid Chromatography (HPLC; Shimadzu Lc8A, Japan). Extraction and quantification of "silymarin" from dried plant samples was done by the valuable protocol of Khan *et al*., (2013).

Statistical analysis: For statistical analysis, each treatment consisted of 10 culture flasks and data was collected from triplicates. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used for comparison among treatment means. However, for chromatographic quantification analysis, data were collected from triplicates and represented as values of mean.

Results and Discussion

Callus induction frequency: TDZ is considered as one of the most potent bio-regulator for indirect regeneration in many plant species (Murthy *et al*., 1998). TDZ induced callogenesis from different explants of many medicinal plants has been reported (Mithila *et al*., 2003), suggesting TDZ as propitious PGR for callus induction than other cytokinins (Thomas, 2003).

Incubation of leaf explants on to MS medium incorporated with different concentrations of PGRs, induced callus in 7 days of culture time (Fig. 1). Negligible (5%) microbial contamination was observed during explant culturing. Explants taken from *In vitro* grown plantlets (Zheng *et al*., 2009) are often more responsive in micropropagation than explants derived from *In vivo* grown plants (Abbasi *et al*., 2010).

In present study, TDZ alone at lower concentrations (2.2 µM) produced optimum callus (73%). Notwithstanding, a decrease in callus formation was observed with increase in levels of TDZ. At concentration ≥ 2.2 µM, pale yellow calli were induced. These observations are in agreement with Radhiks *et al*., (2006). No callus induction response was observed in controls (Fig. 1). Moreover, the callogenic response was enhanced (89%) when MS medium was augmented with TDZ (4.4µM) in combination with Kn (6.6µM) (Fig. 1). Calli developed were soft, friable and greenish white with a diameter of 1.5 cm after four weeks of explant culturing. Similar morphological parameters in callus formation were observed by Mroginski *et al*., (2004). Similarly, a number of reasons can be anticipated for the development of different characteristics in callus cultures like nature, color and biochemical composition, which are usually determined by the composition of the medium; PGR type and concentration along with explants culturing practices (Danya *et al*., 2012) and such variations in calli have been reported in many plant species (Ishi *et al*., 2004).

Shoot organogenesis: The fast, friable and competent calli were transferred into shoot organogenesis media. Highest shoot regeneration frequency (86%) with a maximum number of shoots (26 shoots per explants) was recorded for MS medium supplemented with 11µM TDZ. However, shoot regeneration was induced on a wide range of TDZ concentrations (Figs. 2 and 3). In current report, TDZ alone induced better response than other PGRs tested. Similarly, Banerjee *et al*., (2012) reported TDZ alone as the best PGR for shoot induction in *Hypericum perforatum* L. In our data the highest concentration of TDZ in the medium profoundly enhanced the shoot regeneration frequency. However, a decrease in shoot organogenesis parameters was observed with increase in concentration of TDZ beyond the optimal level. Kumar *et al*., (2012) have reported optimum shoot induction frequency in *Jatropha curcas* at higher concentration of TDZ (9.08 µM). However, Parveen & Shahzad (2010) reported optimum shoot number in *Cassia sophera* Linn at lower concentration of TDZ (2.4µM). Furthermore, Mukhtar *et al*., (2012) concluded from their work that increase in level of TDZ beyond an optimum level has an inhibitory effect on shoot organogenesis. In contrast to our findings, lower values of TDZ have been advocated for higher frequency of shoot organogenesis in many plant species (Raghu *et al*., 2006; Chhabra *et al*., 2008). In our study, calli transferred to MS0 produced no shoots. This is consistent with the results of Yucesan *et al*., (2007) but contrary to the work of Radice & Caso (1997) who found MS0 as an optimum medium for shoot regeneration of *S. marianum*. The stimulating effect of TDZ on shoot organogenesis in many plant species from various explants has been documented (Yucesan *et al*., 2007; Ahmad & Anis 2007; Ismile *et al*., 2011; Erisen *et al*., 2011; Banerjee *et al*., 2012; Wannakrairoj & Tefera, 2012).

Combinations TDZ with NAA produced lower values for shoot organogenesis in current report. Similarly, combination of TDZ with NAA failed to induce shoot organogenesis in *Lycopersicon esculentum* (Osman *et al*., 2010). Findings of Mirici (2004) supported our data. Contrarily, TDZ in combination with other PGRs could be more effective than TDZ alone (Lincy & Sasikumar, 2010). In another report, combination of NAA, Zn and BAP produced best shoot proliferation in *S. marianum* (Gikloo *et al*., 2012).

For shoot elongation, clumps of proliferated shoots were separated into individual shoot (0.2-0.4 cm) and were sub-cultured to medium with similar composition of PGRs (Fig. 4). Significant differences were observed in elongation at different concentrations and combinations of plant growth regulators. Contrary to data on shoot/explant, addition of NAA in to medium containing TDZ increased mean shoot length. Longest mean shoot length (4.1 cm) was observed on medium augmented with 6.6µM TDZ and 4.4µM NAA. Previously, Abbasi *et al*., (2010) observed longest shoots of *S. marianum* on MS medium fortified with BA and NAA. Combination of TDZ & Kn produced smaller shoots (1.2 to 2.3 cm in length). However, NAA showed inhibitory action when applied individually at all concentrations (Fig. 4).

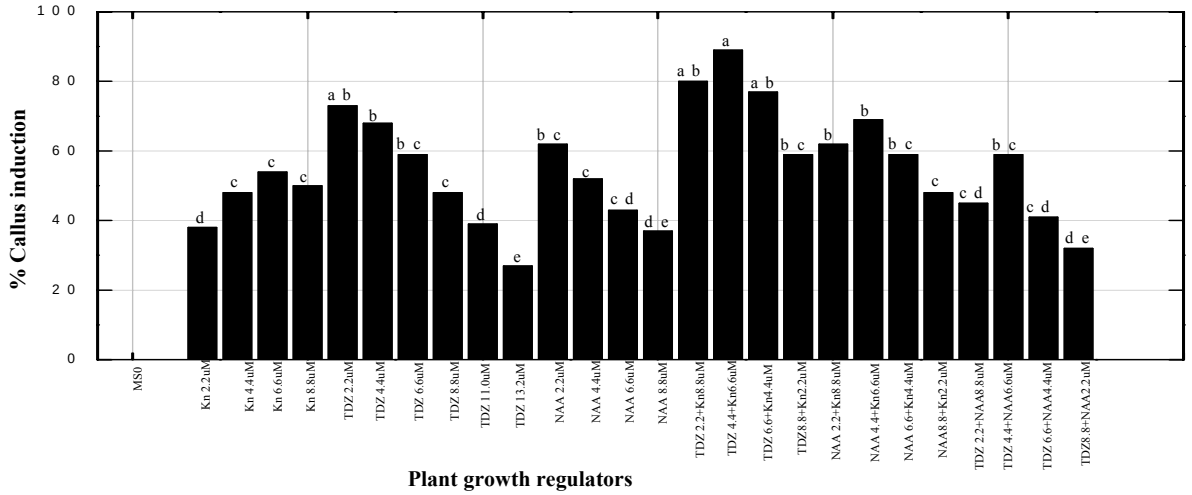

Fig. 1. Data on callus formation parameters in *Silybum marianum* by the application of various concentrations and combinations of TDZ, Kn and NAA. Values are mean of three replicates and observations were recorded after 4 weeks of culture.

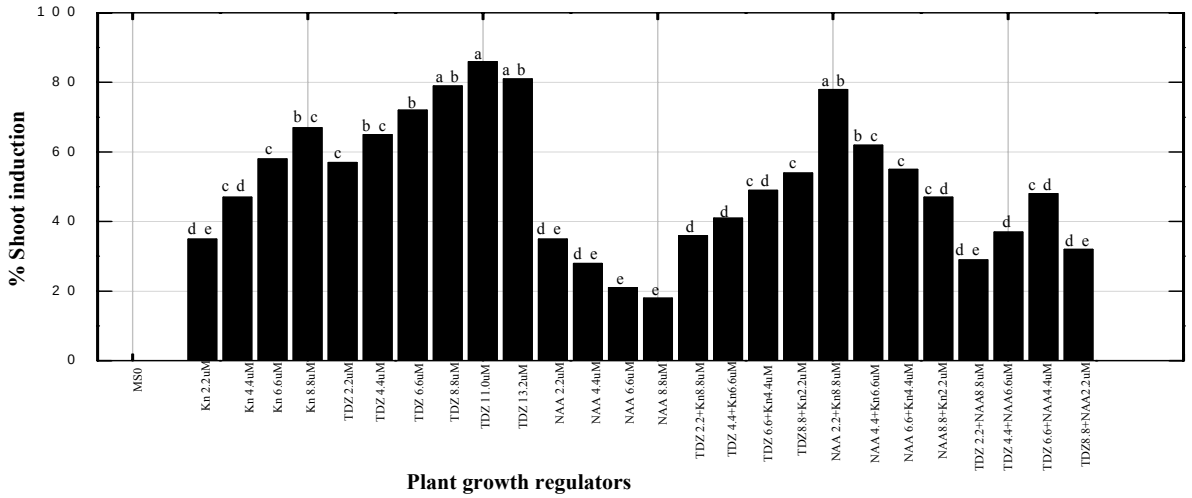

Fig. 2. Data on shoot induction frequency in *Silybum marianum* by the application of various concentrations and combinations of TDZ, Kn and NAA. Values are mean of three replicates and observations were recorded after 4 weeks of culture.

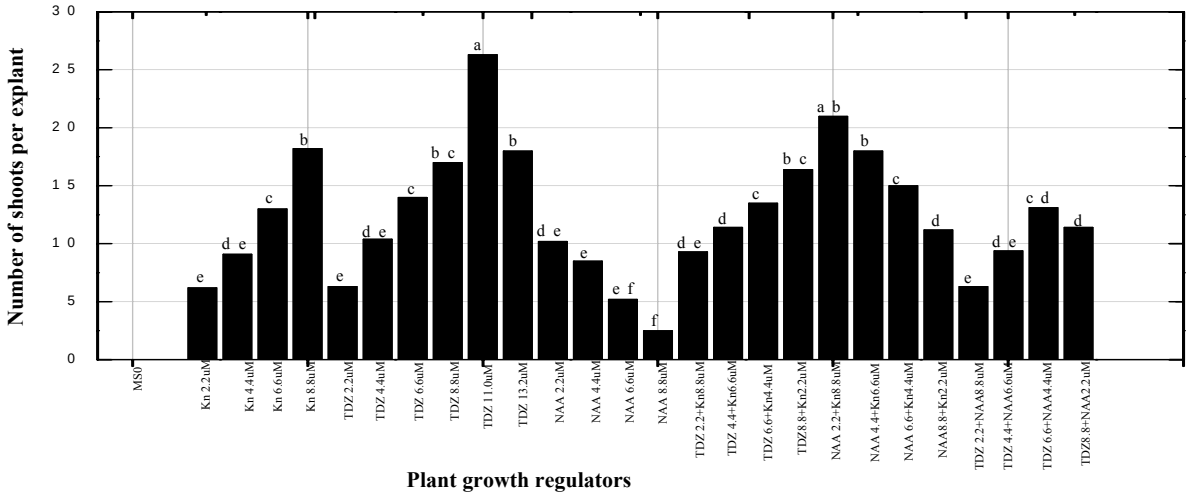

Fig. 3. Data on number of shoots in *Silybum marianum* by the application of various concentrations and combinations of TDZ, Kn and NAA. Values are mean of three replicates and observations were recorded after 4 weeks of culture.

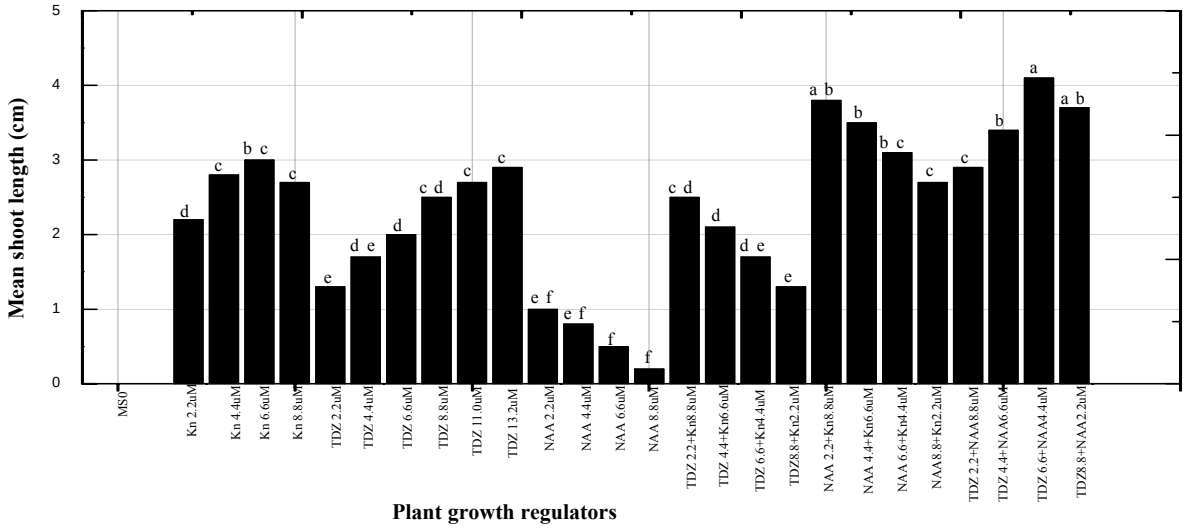

Fig. 4. Data on shoot length (cm) in *Silybum marianum* by the application of various concentrations and combinations of TDZ, Kn and NAA. Values are mean of three replicates and observations were recorded after 4 weeks of culture.

The pivotal role of TDZ in profuse shoot organogenesis in present work can be ascribed by its stimulating effect on plant morphogenesis in the *de novo* synthesis of auxins and cytokinins by increasing the level of indigenous precursors for their profound production (Li *et al*., 2012). Furthermore, TDZ is supposed to inhibit cytokinin oxidase, the enzyme responsible for the inactivation of cytokinin, thereby increasing the levels of endogenous cytokinins (Nikolic *et al*., 2006). This explains the higher activity of TDZ in achieving highest shoot organogenesis in our study.

Rooting and acclimatization: Elongated shoots were transferred to MS0 and MS medium containing several concentrations (0.2, 0.4, 0.8, 1.6 or 3.2 µM) of NAA or IAA (Table 1). IAA showed more effective results than NAA by promoting highest rooting frequency (87%) at 0.4 µM IAA with mean number of roots (3.2 roots) and mean root length (3.4 cm). However, increasing concentrations of both NAA and IBA resulted in a linear decrease in rooting; maximum inhibition of the rooted shoots was observed for NAA at 3.2 µM. In present study reduced levels of auxins considerably induced roots as generally reported for root induction and proliferation in many herbaceous plants (Victorio *et al*., 2012). Recently, Gikloo *et al*., (2012) has reported combination of activated charcoal with IBA (0.25mg l^{-1}) as best medium for root development in *S. marianum*. But previously, Abbasi *et al*., (2010) induced optimum rooting of *S. marianum* on MS0. Finally the rooted shoots were transferred to sterilized vermiculite and kept under growth room conditions for two weeks prior transfer to pots. The survival rate of rooted shoots was 73% through the hardening off process.

Silymarin content: Callus, *In vitro* shoots, micropropagated plantlets and growth room potted plantlets were collected at various growth stages for HPLC analysis to determine the content of Silymarin (Fig. 5). To standardize the fingerprints of tissue cultured materials through HPLC, similar peaks present in all samples were denoted as "common peaks" for silymarin production in *S. marianum*. The chromatographic data along with silymarin standard (Sigma) representing valuable information based on the relative peak area and retention time for assessment and quantification of silymarin in all *In vitro* and *In vivo Silybum* samples.

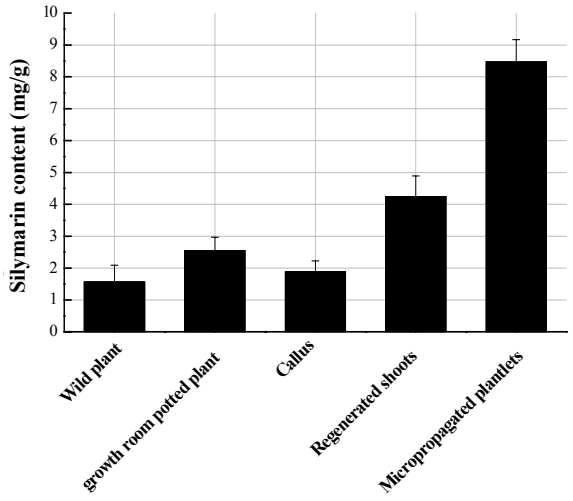

Fig. 5. Data on silymarin content (mg/g) in regenerated plant tissues and wild grown plantlets of *Silybum marianum*. Values are mean of three replicates.

8.47 mg/g silymarin was detected in regenerated plantlets. Differentiated tissues possess higher levels of silymarin than callus (Fig. 5). Our results demonstrate an ascending variation of silymarin from callus culture to plantlets development depicting the influence of growth pattern on silymarin production in the *In vitro* regeneration of this medicinally important herb.

Table 1. Data on root formation parameters by the application of different concentrations of indole acetic acid (IAA) and α-naphthalene acetic acid (NAA). Values are mean of three replicates and observations were recorded after 4 weeks of culture.

Conc. (µM)	NAA IAA rooting (%)		NAA IAA No. of roots/shoot		NAA IAA root length (cm)	
0.2	52.45^{ab}	73^{ab}	2.5^{ab}	2.9^{ab}	3.1^{a}	2.8^{ab}
0.4	61^{a}	87.32^{a}	2.9^{a}	3.2^{a}	2.7^{ab}	3.4^{a}
0.8	46^{b}	61^{b}	2.3^{ab}	2.7^{ab}	2.2^{ab}	3.1^{a}
1.6	33^{bc}	53^{bc}	1.9^{bc}	2.1^{bc}	1.9^{bc}	2.7^{bc}
3.2	13^{c}	44.53^{c}	1.3^{c}	1.8^{c}	1.4^{c}	1.9^{c}

Growing conditions have a direct relationship with accumulation of secondary metabolites (Naz *et al.,* 2013) governed by stress conditions, which consequently influence the metabolic pathways responsible for the accumulation of the related natural products (Nikolova & Ivancheva 2005; Abbasi *et al.,* 2011). In case of silymarin, TDZ supplementation has shown significant effect on its accumulation in current study. Biosynthesis of silymarin involves activity of phenylalanine ammonia lyase (PAL) in phenylpropanoid pathway (Koksal *et al*., 2009). Furthermore, TDZ treatment is reported to enhance the levels of secondary metabolites via modifications of plant growth and development, since its biological activity is generally higher than most of the plant growth regulators when applied to *In vitro* cultures (Liu *et al*., 2007). Our results are comparable with the findings of Sherif *et al*., (2012); they observed considerable silymarin content in multiple shoot cultures of *S. marianum* by different biotic elicitors.

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