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QUALITATIVE AND QUANTITATIVE ANALYSIS OF THAUMATIN LIKE PROTEINS (TLPS) IN *IN VIVO* AND *IN VITRO* CULTURES OF GYMNEMA *SYLVESTRE*

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Plant produces different pathogenesis related proteins as a defence mechanism against pathogenic conditions and in adaptation to stressful environment. TLPs are a group of proteins from PR 5 category which have shown homology with thaumatin from *Thaumatococcus daniellii* and have shown antibacterial activity. Exploration of PR proteins from *in vivo* plant materials (leaves) and from *in vitro* (cell suspension and shoot) cultures of *G. sylvestre* using Western blot and ELISA techniques has been reported for the first time. Western blot analysis showed a prominent band at 22 kDa which correspond to the TLPs of *G*ymnema *sylvestre* which compared with standard TLPs. ELISA analysis showed higher quantity of TLPs (2.067 µg/3g) in *in vivo* plant materials as compared to shoot cultures (1.729 µg/ 3g) and cell suspension cultures (1.721 µg/3g).

Keywords- ELISA, *G. sylvestre*, SDS- PAGE, TLPs, Western blot

The production of pathogenesis related (PR) proteins are a common strategy of plant cells or plants against various stress factors (Edreva 2005, Sinha *et al*. 2014). These proteins are produced in plants as a defence mechanism against pathogenic conditions and in adaptation to the stressful environment (Edreva 2005, Sinha *et al*. 2014). The PR proteins are triggered by the attack of viruses, bacteria and fungi (Van Loon 1985, Lamb *et al.* 1989) and similarly produced by the application of chemicals that mimic the effect of pathogen attack (Delaney *et al.* 1994, Xu *et al.* 1994). These PR proteins are also produced constitutively in different organs during certain developmental stages (Lotan *et al.* 1989, Linthorst *et al.* 1990). These proteins are strongly induced by infections and therefore listed as PR proteins and have been classified into various families based on the shared sequence homology (Van Loon *et al.* 1994). Such proteins have been observed for the first time in the leaves of *Nicotiana tabacum* infected with tobacco mosaic virus (Van Loon and Van Kammen 1970). Thereafter, PR proteins have been detected in various plant species and localized in almost all plant organs including leaves, stems, roots and flowers. Their maximum abundance is observed mainly in the leaves where they amount to 5-10 % of total leaf proteins (Van Loon *et al.* 1999). Initially, Kitajima and Sato (1999) have grouped PR proteins in 5 major groups which were further increased after identification of new PR proteins. Currently 17 PR proteins families are known and these are classified in different groups based on their amino acid sequence similarities, enzymatic activities and/ or other biological properties (Van Loon *et al.* 1999, Buchel and Lithorst 1999, Van Loon 2006). PR-5 proteins have amino acid homology with *Thaumatococcus daniellii*, a sweet tasting protein, from South Africa (Daniell *et al.* 1852) and are known as thaumatin-like proteins (TLPs).

An earlier study has reported antibacterial activity for Gymnema *sylvestre* (Khanna and Kannabrian 2008, Yogisha and Raveesha 2009, Sinha *et al*. 2010, David and Sudarsanam 2013) where it was postulated that this activity could be because of gymnemic acid. It has also been reported that similar antibacterial activity is shown by PR-5 protein (Sinha *et al*. 2014) like thaumatin-like proteins (TLPs). So in the present study, an attempt has been made to explore PR proteins from non pathogenated *in vivo* plant materials (leaves) and from *in vitro* (cell suspension and shoot) cultures of Gymnema *sylvestre* using denaturing

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polyacrylamide gel electrophoresis (SDS PAGE), Western blot and enzyme linked immunosorbent assay (ELISA) techniques.

MATERIALS AND METHODS

Sample preparation

i. In vivo cultures

Leaf sample (3 g) was collected from *in vivo* grown (Approximately 4 year old) plant of from Department of Botany, Savitribai Phule Pune University, Pune- 411007, between June and August 2015. Collected plant sample was identified and authenticated from Botanical survey of India, Western circle Pune.

ii. In vitro cultures

Approximately 3 g of shoots (with leaves) grown on MS + BAP, KIN, NAA (4.44, 2.32, 2.69 µM) (eight week old) (Zimare and Malpathak 2016) and cell suspension cultures grown on MS + 2, 4-D, BAP (4.52, 4.44 µM) (four week old) (Zimare *et al.* 2017) were selected which showed higher biomass production in the study duration. The friable calli obtained on MS (Murashige and Skoog medium, 1962) + 2, 4-D (4.52 M), which showed the highest callus proliferation, used for the initiation of cell suspension cultures (Zimare *et al.* 2017).

Protein extraction

The samples were homogenized with 5 ml of phosphate buffer (pH 7.5) having 0.053 µl - mercaptoethanol and 67 mg PVP. Extracts were centrifuged at 10000 rpm for 20 min and supernatants were collected. Protein concentration in the extracts was determined using Bradford assay (Bradford 1976) and further processed for qualitative and quantitative investigation.

Qualitative investigation of TLPs

a. Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis (SDS PAGE)

Obtained protein sample (15 µg) was mixed with equal amount of loading dye (bromophenol blue 2X dye) and was boiled at 95°C for 5 minutes prior to addition in to the well of SDS- PAGE gel. SDS-PAGE was performed on 4 % stacking gel and 12 % resolving gel as described by Laemmli (Laemmli, 1970). After electrophoresis, the gel was stained by coomassie brilliant blue R - 250 for 30 min and thereafter, destained with methanol : glacial acetic acid : distilled water (4 : 1 : 5 v/v). The quality of protein band was compared to the standard thaumatin protein (Sigma, USA) and molecular weight markers (Mid range 14-80 kDa, SRL, India).

b. Western blot

The separated proteins on the gel were transferred onto a nitrocellulose membrane. The nitrocellulose membranes were blocked with 1 % bovine serum albumin (BSA) prepared in tris-buffered saline (TBS) containing 0.05 % Tween-20 for 1 hour at room temperature (RT). After blocking, the nitrocellulose membrane were incubated with primary (Pru p 2 with 1:5000 concentration) and secondary antibodies [goat anti-rabbit (h+l) IgG labbled with HRP] with 1: 5000 concentration for 1 h, respectively. The blots were washed extensively with washing buffer (Invitrogen, WB01) containing 0.05 % Tween 20 after every step. The blot was then treated with TMB (3,3'5,5' Tetramethylbenzidine, Sigma, T0440) solution in dark to visualize the band. The experiment was repeated thrice.

Quantitative investigation of TLPs

a. Enzyme-linked immunosorbent assay (ELISA)

For ELISA, 96-well flat-bottom plates (Nunc, 442404) were used. The plates were coated with different concentrations of protein (10, 15, 20 µg), diluted with coating buffer (carbonate bicarbonate buffer, Sigma, C3041-50CAP) for overnight at 4°C. Thereafter, plates were blocked with phosphate buffer saline (PBS, Sigma, P3563-10PAK) containing 0.05 % Tween-20 and 2 % BSA for 1 h at RT. After

blocking the plates were washed three times with PBS containing 0.05 % Tween-20. The plates were then incubated for 2 h at RT with primary antibody (Pru p 2 with 1:5000 concentration) diluted in blocking buffer. The plates were washed again and incubated with the secondary antibody (goat anti-rabbit (h+l) IgG labbled with HRP) diluted at 1:5000 concentration with 2 % BSA and 0.05 % PBS-T for 1 h at RT. Thereafter, IgG reactivity was detected by adding TMB (3,3'5,5' Tetramethylbenzidine, Sigma, T0440) in dark, followed by addition of stop solution (Sigma, A5852-100ML). The colour intensity was measured on Thermo scientific multiscan EX ELISA reader at 450 nm. All the experiment was performed in triplicate.

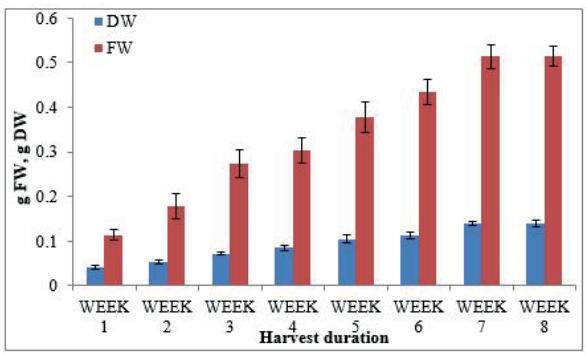
Statistical analysis

All the experiments were carried out in triplets and the data were expressed as means ± SD.

RESULT AND DISCUSSION

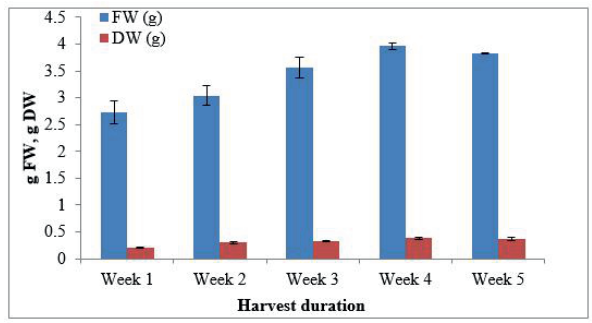
The four and seven weeks old cell suspension and shoot cultures grown on 2,4-D, BAP (4.52, 4.44 µM) and BAP + KIN + NAA (4.44 + 2.32 + 2.69 µM) were selected for the present experiment and the cultures were chosen on the basis of their growth patterns (Figure 1 and 2**)**. We selected *in vitro* cultures of stationary phase, which are known to have higher content of different proteins. Elsewhere *in vitro* cultures have shown increase in the plant protein content during the exponential phase, and were highest at the end of the stationary phase (dos Santos *et al*. 2010).

Qualitative investigation of TLPs from *G. sylvestre* was done by SDS-PAGE and Western blot technique. The protein bands obtained were compared with standard thaumatin as a positive control, where it confirming its presence in the sample. The protein separation of *in vivo* material and *in vitro* cultures were analyzed by Coomassie brilliant blue R-250 stained SDS-PAGE (Figure 3 a) which is an easy method and allows protein detection from plant samples. We have also employed the Western blot (or immunoblot) technique widely used and accepted analytical technique which measures proteins in a biological sample, and frequently used to detect the pathogenesis-related proteins (Elvira *et al*. 2010; Xu *et al*. 2014). In the present study, SDS PAGE was followed by Western blot analysis which showed a prominent band at 22 kDa (Figure 3 a and b) which may correspond to the TLPs of *G. sylvestre*. TLPs have been categorised into two types: one is having molecular weight ranging from 22 kDa to 26 kDa, and the other having molecular weight of 16 kDa (Roberts and Selitrennikoff 1990). In the present investigation, TPLs from *G. sylvestre* belonged to the first type having molecular weight of 22 kDa. The PR proteins are usually known to be produced by various pathogens, such as bacteria, viruses, fungi (Van Loon 1985, Rigden and Coutts 1988, Lamb *et al*. 1989) and in response to certain chemicals which mimic the effects of pathogen infections



Data represents mean values ± SD of three replicates.

Figure 1: Growth kinetics of multiple shoots cultures of *G. sylvestre* grown on BAP, KIN, NAA (4.44, 2.32, 2.69 µM) for eight weeks



Data represents mean values ± SD of three replicates.

Figure 2: Growth kinetics of cell suspension cultures of *G. sylvestre* grown on 2, 4-D, BAP (4.52, 4.44 µM)) for five weeks

Table 1: Concentrations of TPLs from *in vivo* plant materials and from *in vitro* cultures of *G. sylvestre* by ELISA technique.

No.	Culture system used (10 µg)	Conc. (µg/ 3g)
1	*in vitro* cell suspension	1.721 ± 0.02
2	*in vitro* shoot	1.729 ± 0.01
3	*in vivo* leaves	2.067 ± 0.02

* Data represents mean ± SD

(Delaney *et al*. 1994). In addition to the induction of PR proteins in response to the stress some of the PR proteins are constitutively expressed in some plant parts during certain developmental stages (Linthorst *et al*. 1990). Hence, the selection of plant cultures at specific growth or developmental stage is essential. This could be the reason for the presence of PR proteins in non-pathogenated *in vivo* plant materials and in *in vitro* cultures of *G. sylvestre*, which have been detected in SDS-PAGE and Western blot analysis (Figure 3 a and b).

TLPs from *in vivo* plant materials and *in vitro* cultures of *G. sylvestre* were quantified using ELISA technique which is widely used for quantitative detection of PR proteins (Muñoz-García *et al*. 2013). *In vivo* plant material demonstrated higher quantity of TLPs (2.067 µg/3g), as compared to shoot cultures (1.729 µg/ 3g) and cell suspension cultures (1.721 µg/3g) (Table 1) by ELISA. Le Bourse *et al*. (2011) have used ELISA for quantification of TLPs from grape juice and reported 89.0 ± 13.9 mg/ml, 85.8 ± 8.5 mg/ml, 25.8 ± 4.1 mg/ml, 1.1 ± 0.1 mg/ml, 0.8 ± 0.1 mg/ml TLPs respectively for grape juice (CH09), grape juice (PM09), base wine (CH08), Champagne (CH08) and Champagne (CH06). A difference in the quantity of TLPs was observed in our study as compared to the study of Le Bourse *et al*. (2011) which could be because different plant parts and plant system used in our study. Additionally, they have purified proteins using HPLC, mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy, whereas, we have used only crude protein extracted from *in vivo* and *in vitro* cultures of *G. sylvestre*.

The PR proteins are constitutively expressed in some plant parts (Van Loon 1985, Linthorst *et al*. 1990). This could be the reason for its detection in ELISA in non-pathogenated *in vivo* leaves and *in vitro* cultures. The TLPs have been observed in non-pathogenated tissue and cultures of *G. sylvestre* and similar results have been seen in *in vivo* plant materials of tomato, potato and beans (Sudisha *et al.* 2012,

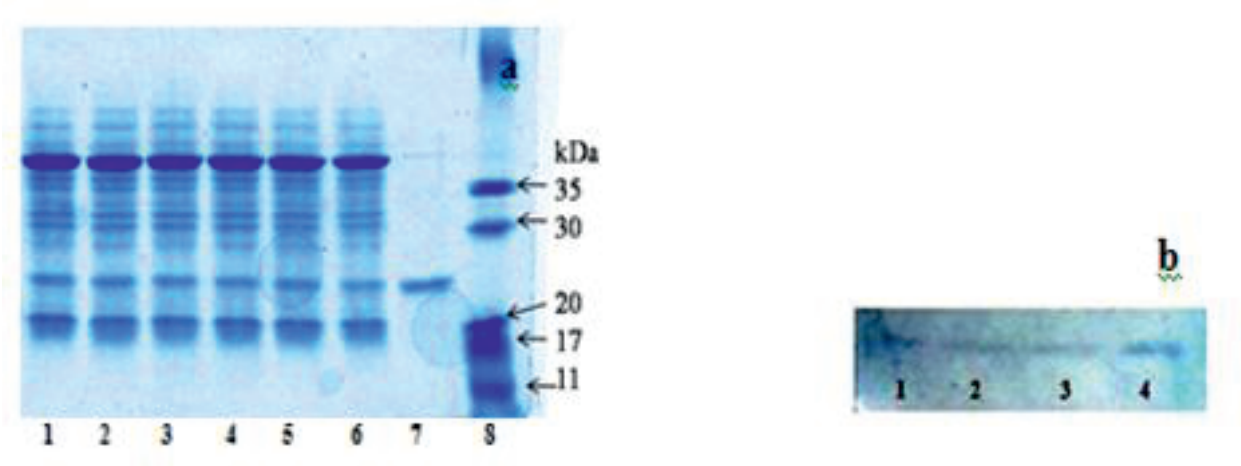


Figure 3: SDS PAGE (a) and Western blot (b) analysis of TPLs from *in vivo* plant materials and *in vitro* cultures of *G. sylvestre.* **(a)** Lane 1, 2 - cell suspension culture; lane 3, 4 - shoot culture; lane 5, 6 - *in vivo* leaves; lane 7 - standard thaumatin, lane 8- marker. **(b)** Lane 1 - cell suspension culture; lane 2 - shoot culture; lane 3 - *in vivo* leaves; lane 4 - standard thaumatin

Tuzun 2001). Presence of PR proteins in non pathogenated plant samples was observed showing a resemblance to the study reported by Fraser (1981) and Hanfrey *et al*. (1996) who have also seen the formation of PR proteins in non pathogenated leaves of tobacco and *Brassica* species, respectively. Pathogen infection or attack produces various signalling molecules like salicylic acid (Delaney *et al*. 1994), ROS (Chamnongpol *et al*. 1998) and methyl jasmonate (Xu *et al*. 1994) which in turns upregulates various defence related genes, such as PR protein genes (Sinha *et al*. 2014). These signalling molecules may be present in the healthy and non infected tissues also. Our observations have shown production of different quantities of TLPs which could be because of different concentrations of ethylene present at different developmental stages. Production of ethylene in different plant tissue is dependent upon the respective developmental stages and the plant organ itself (Sinha *et al*. 2014). These could be one of the reasons of finding higher concentrations of TLPs in *in vivo* plant materials and shoot cultures of *G. sylvestre*. Apart from the signalling molecules and environmental stimuli, PR proteins are also produced due to internal developmental stimuli of the plants (Sinha *et al*. 2014). Different concentrations of TLPs were seen in *in vivo* plant materials, which were higher than *in vitro* cultures of *G. sylvestre*. This could be because expression of PR proteins is cell, tissue and organ specific and they are also developmentally expressed (Linthorst *et al*. 1990). The above reasons may have induced TLPs in the materials used in the present study.

Our observations have shown that leafy tissue i.e. *in vivo* plant material and *in vitro* shoot cultures have higher quantity of TLP as compared to the cell suspension cultures, which showed concurrence with the results of Van Loon (1985) and Gette and Marks (1990). The PR proteins are known for its localization in all the plant parts including vegetative and reproductive parts, however being prominently or abundantly present in leaves only. Expression of most of the PR proteins like TLPs is accelerated by ethylene in the abscission zone of leaves, which could be a reason for the higher quantity of TLP in leafy tissue (*in vivo* leaves and *in vitro* shoot cultures). Additionally, different chemicals like salicylic acid, inorganic salts are also inducers of PR proteins such as TLPs (Edreva, 2005). Several PGRs like BAP, KIN, 2, 4-D and NAA) have been used in different concentrations and combinations for raising in *vitro* cultures of *G. sylvestre*. These PGRs are also known to induce TLPs (Edreva 2005) and could be reason for its presence in the cultured tissues, and therefore detected by SDS-PAGE, Western blot and ELISA techniques.

CONCLUSION

The present study shows that non pathogenate *in vitro* cultures and *in vivo* plant materials could serve as a source of TLPs. Traditionally *G. sylvestre* is mainly used to treat diabetes and its related complications but, the present data have unravelled its significance in the production of TLPs where it opens up a new area of research.

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