Summary

*P. rosea* syn. *Indica* belong to the family of *plumbaginaceae*, is an important medicinal plant, cultivated widely in India. The roots of these plant are generally used for medicinal purposes mainly as diuretic, germicidal, vessicant, and abortifacient. It is also used for anaemia, diarrhea, leprosy and common wart. The bark of the root contains orange yellow pigment named plumbagin, a crystalline substance, belongs to the class of naphthoquinone. Its chemical structure is 5-hydroxy 2-methyl 1,4-naphthoquinone. Apart from *P. rosea*, *P. zeylanica*, *P. europea*, *Drosera* and *Drosophyllum* also contains plumbagin. The most exploited source of plumbagin is, of course, *P. rosea* roots. The roots contain 0.9mg/g D.Wt. of plumbagin in the roots. These plants grow very slowly and the roots suitable for plumbagin extraction can be obtained only after several years of growth. The productivity of the plant is also rather poor.

The focus of the present study was to develop alternative strategies to obtain plumbagin. The tissue culture of *P. rosea* for micropropagation has been studied. However, reports on plumbagin accumulation is scanty. The tissue culture route to obtain plumbagin appears to be attractive and technically feasible. There are several reports on various treatments to stimulate the secondary metabolism in plant cells. The major objectives of this study are,
1. To optimize the media conditions for growth of callus culture of *P. rosea* and plumbagin accumulation.
2. Studies on hairy root culture of *P. rosea*.
3. To study the influence of various permeabilizing agents such as CTAB, DMSO, Triton X-100 and Chitosan on release of the metabolite into the bulk medium.

(1) To study the effect of various biotic elicitors prepared from bacterial and fungal cultures for enhanced accumulation of plumbagin.

(2) To apply combination of treatment methods for synergistic influence on plumbagin accumulation.

(3) To optimize selected treatment methodologies for enhancement of plumbagin production.

To achieve the above aims, systematic studies have been planned to develop callus and hairy root cultures of *P. rosea*.

The callus cultures of *P. rosea* were developed in Murashige and Skoog, Schenk and Hildebrandt and Gamborg’s medium. The composition of growth hormones were standardized with various concentrations of auxins (IAA, NAA, 2,4-D) and cytokinins (BAP and Kinetin). It was observed that maximum biomass accumulation was developed in MS medium containing 1 mg/L IAA, 0.5 mg/L of NAA and 0.3 mg/L of BA. It was also noticed that 5 fold increase in dry weight was obtained in 30 days of culturing and the plumbagin content has doubled during this period. Embryogenic callus cultures were obtained after regular subculturing and screening for more than two years. The plumbagin content in embryogenic callus cultures were found to be double to that of the non-embryogenic callus on 30th day of incubation. Cent per cent shoot regeneration was obtained using 1.0 mg/l IAA and 1.0 mg/l BA. The doubling time of the callus was found to be 9 days in MS medium, 14 days in B5 medium and 18.5 days in SH medium and accordingly highest specific growth rate of 0.0767 day\(^{-1}\) was noticed in MS medium. The callus cultures were maintained for a period of 5 years by
subculturings every 2 weeks and incubated at 25± 2°C at 10/14 photoperiod. An attempt has been made to develop hairy root culture of *P. rosea* by infecting the shoots and leaf discs. The hairy roots obtained were analyzed for plumbagin content. It was noticed that both doubling time of the hairy roots was more and plumbagin content was found to be lower than callus cultures. Hence, further studies were confined to with callus cultures only.

The accumulation of plumbagin at various dosage levels of permeabilizing agents such as CTAB, DMSO, Triton X-100 and Chitosan were studied. Both intracellular and extracellular plumbagin was estimated. It was noticed that cell damage was very severe in case of chitosan, and CTAB whereas DMSO and Triton-X-100 at lower dosage level and shorter contact time could alter the cell membrane integrity effectively, to release the metabolite into the medium without damaging the cells. It was observed that CTAB and Chitosan cannot be used as permeabilizing agents but very effective to release the metabolites.

*P. rosea* cells were challenged with elicitor compounds prepared using fungal and bacterial cultures. The fungal cultures used were *Aspergillus niger*, *Rhizopus nigricans* and the bacteria used was *Bacillus cereus*. *Saccharomyces cerevisiae* was also tried for elicitation purposes. Dose response profiles were generated with each elicitor. In addition to hydrolysed biomass, spent culture media was also used as elicitors. Among all the microbial strains, the elicitor prepared from Bacillus was given lowest response (39.4-62.98%). The mycelial hydrolysate of *R. nigricans* has exhibited 200 to 236 % higher accumulation of the metabolite. It is significant to note that the spent culture medium of *A. niger* has found to be very effective as elicitor. The accumulation of metabolite was nearly 3 fold over control.
The synergistic effect of permeabilization, elicitation, immobilization and *in situ* product removal, when used in combination were studied. In one set of experiments, the cells of *P. rosea* were immobilized in sodium alginate and subsequently permeabilized with 0.001 ppm CTAB or with 0.01% (v/v) DMSO for short duration. The treated immobilized cells were cultured in fresh medium containing XAD-2, a nonionic adsorbent. Parallelly, experiments were carried out using individual treatments only. It was noticed that the combination of treatments, immobilization and resin addition has enhanced the plumbagin accumulation by 230 percent over control (9.83 mg/g D.Wt.). Similarly, experiments were carried out with elicitation instead of permeabilization. The elicitor used was derived from *A. niger* or *R. nigricans* (1.0% v/v). It was found that multiple treatment strategies (immobilization, elicitation and resin addition) has resulted in nearly 200 percent higher yield of plumbagin over control. Similar results were also obtained when the cells were treated with 1.0% (v/v) *R. nigricans* derived elicitors.

From these studies, it can be concluded that the increase in metabolite yield was found to be 200 to 250 percent higher than the control when the cells were subjected to either individual treatment such as permeabilization and elicitation or combination of treatments. This has led us to speculate that in case of stress related metabolites (such as naphthoquinones) the synergistic effect of combined treatment is negligible due to saturation effect of the treatment used and beyond a levels of these stress applied to stimulate the product accumulation, the cell response was poor. The threshold level of stress to the cells to trigger the enhanced product accumulation can be achieved either by individual treatment or by sequential treatments. In all
the treatment strategies we have attempted, the increase in metabolite yield was found to be nearly 3 fold irrespective of the treatment used.

The present research activity conclusively demonstrates the potential of callus cultures of *P. rosea* as a source of plumbagin. Employing various treatments viz. elicitation, permeabilization and immobilization could enhance the product accumulation in the callus cultures. The same procedure could be applied for the production of compounds belonging to the group of naphthoquinones as most of these are found produced during stress conditions. Further work pertaining to large scale culturing is needed before evaluating this methodology on techno-economical basis.