# ISOLATION, PURIFICATION AND FUNCTIONAL STUDY OF ARABINOGALACTAN PROTEIN FROM GROUNDNUT

**A THESIS**

*Submitted in partial fulfilment of the requirements for the award of the degree of*

DOCTOR OF PHILOSOPHY

*in*

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*by*

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# INDIAN INSTITUTE OF TECHNOLOGY ROORKEE

## CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **ISOLATION, PURIFICATION AND FUNCTIONAL STUDY OF ARABINOGALACTAN PROTEIN FROM GROUNDNUT** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2002 to February 2008 under the supervision of Dr. Ramasare Prasad, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Ashish Deep

**(ASHISH DEEP GUPTA)**

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

(Ramasare Prasad)
Supervisor

Dated: 20.2.2008

The Ph.D. Viva-Voce Examination of Mr. Ashish Deep Gupta, Research Scholar has been held on ....20Th Oct, 2008

Signature of Supervisor

20.10.08

Signature of External Examiner

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Ashish Deep Gupta

# LIST OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| °C | °Centigrade |
| 2,4-D | 2,4-Dichlorophenoxyacetic Acid |
| AG | Arabinogalactan |
| AGP | Arabinogalactan Protein |
| AGP-AmB | Arabinogalactan protein-Amphotericin-B conjugate |
| Agr | Arginine |
| Ala | Alanine |
| AmB | Amphotericin-B |
| Asn | Aspargine |
| Asp | Aspartate |
| BAP | 6-Benzylaminopurine |
| CFU | Colony Forming Unit |
| cm | Centimeter |
| Cys | Cysteine |
| EDTA | Ethylene Di Amine Tetra Acetate Ion |
| fig. | Figure |
| g | Gram |
| Gly | Glycine |
| h | Hour |
| His | Histidine |
| Hyp | Hydroxy proline |
| Lys | Lysine |
| mg | Milligram |
| mg/L | Milli Gram / Litre |
| mg/ml | Milli Gram / Milli Litre |
| min | Minute |
| ml | Milli Litre |
| mM | Milli Mole |
| MS media | Murashige and Skoog Media |
| NAA | α-Naphthalene Acetic Acid |
| PAGE | Poly-Acrylamide Gel Electrophoresis |
| PBS | Phosphate Buffer Saline |
| Pro | Proline |
| SDS | Sodium Dodecyl Sulphate |
| sec | Second |
| SEM | Scanning Electron Microscopy |
| SE | Standard Error |
| Ser | Serine |
| Thr | Threonine |
| v/v | Volume / Volume |
| µg | Microgram |
| µg/ml | Micro Gram / Milli Litre |

# CHAPTER-1

# INTRODUCTION

Cell surface macromolecules play a significant role in the growth and development of almost all organisms from microbes to higher animals and plants. Besides, they were also found to be of great therapeutic importance. Among them, glycoconjugates (glycoproteins and proteoglycans) have drawn more attention due to their diverse biochemical and medical potential (Ooi and Liu, 2000). The search for a novel glycoconjugate originates from the main fact that most of the commonly used antitumor drugs, chemicals and radiotherapy which are found to be cytotoxic to the cancerous cells are also toxic to normal cells (Kim *et al.*, 1996; Borchers *et al.*, 1999). Thus the discovery and identification of new safer drugs, without severe side effects, has become an important area of research in biomedical sciences (Han *et al.*, 2001). The glycoconjugates, mainly the polysaccharide-protein complexes are found to be more suitable alternative because of its high water solubility, immunostimulatory and antitumor activities. During past two decades, a number of polysaccharides and proteoglycan/glycoproteins with immunostimulatory and antitumor activities have been isolated from diverse sources such as mushrooms (Han *et al.*, 1999; Kim *et al.*, 2003), lichen (Zhang *et al.*, 2002) and plants (Hauer and Anderer, 1993; Classen *et al.*, 2000; Classen *et al.*, 2006; Duan *et al.*, 2003).

One such group of molecules, which play important role in cell growth and development processes are natural plant derived glycoconjugates known as Arabinogalactan proteins (AGPs) and like molecules. AGPs are a group of proteins which contain both carbohydrate and protein as their constituents and are collectively known as Hydroxyproline rich glycoproteins. The protein backbone of AGP is typically hydroxyproline rich and the carbohydrate chain is arabinogalactan (AG) type which primarily consists of galactose and arabinose which are covalently attached to the protein backbone. The AG chain is usually a branched polysaccharide consisting

of (1,3)-β-D-galactan backbone, having (1,6)-β-galactan side chains, which are terminally modified by arabinose with some exceptions where other less-abundant sugar may be present (Gasper *et al.*, 2001; Showalter, 2001). On the basis of protein backbone, AGPs can be classified into two broad groups designated as "classical" and "non-classical". The classical AGP protein back bone has a predicted domain structure and typically rich in hydroxyproline, alanine, serine, threonine and glycine. The classical AGP's core is unique in their organization, comprising of an N-terminal secretion signal, hydroxyproline rich glycosylation domain and a C-terminal hydrophobic sequence with a site for GPI-anchorage (Du *et al.*, 1996b; Schultz *et al.*, 1998). They are attached to the membrane via a GPI anchor and during GPI anchor addition the rest of the C- terminal amino acids are removed. On the other hand non-classical AGPs do not have GPI anchor and their protein backbone differ from classical protein core in many ways as they are relatively Hyp poor, rich in Asn and Cys (Schultz *et al.*, 2000, 2002; Johnson *et al.*, 2003; Mashiguchi *et al.*, 2004). AGPs are widely distributed throughout the plant kingdom from bryophytes to angiosperms (Showalter, 1993; Nothnagel, 1997; Tischer *et al.*, 2002; Letarte *et al.*, 2006; Tang *et al.*, 2006; Pereira-Netto *et al.*, 2007). AGPs as a group are widely distributed in various organs and tissues. At organ level they are found in stems, leaves, roots, floral parts, vascular tissues and seeds (Nothengel, 1997; Coimbra and Duarte, 2003; Motose *et al.*, 2004; Huang *et al.*, 2007; Nguema-Ona *et al.*, 2007). AGPs are found in many tissues, and are especially abundant and well documented in xylem, stylar transmitting tissues and cell suspension culture. Inside cell they have been found in extracellular space, plasma membrane, and the cytoplasmic organelles (Fincher *et al.*, 1983; Komalavlas *et al.*, 1991; van Hengel *et al.*, 2004; Coimbra *et al.*, 2007).

AGPs are found to be highly polymorphic molecules and implicated to play roles in plant growth and development including cell fate, cell proliferation, cell expansion, sexual reproduction, programmed cell death (Gao and Showalter, 1999; Majewska and Nothngel, 2000; Chaves *et al.*, 2002; Lee *et al.*, 2005; Yang and Showalter 2007), cell signaling and somatic embryogenesis (Knox, 1995; Thomson and Knox 1998; Samaj *et al.*, 1999; van Hengel *et al.,* 2001; Letarte *et al.*, 2006; Lamprot *et al.*, 2006). Expression level of AGPs have been found to be developmentally regulated and also depend on biotic and abiotic stress, wounding, pathogen invasion, heat shock and phytohormones (Li and Showalter, 1996; Gilson *et al.*, 2001; Park *et al.*, 2003; Sun *et al.*, 2004). Because of its significant role in cell growth and development, AGPs from various important crops have been isolated, structure determined and a number of genes have been cloned and characterized (Sommer-Knudsen *et al.*, 1997; Serpe and Nothnagel, 1999; Schultz *et al.*, 2000; Gaspar *et al.*, 2004). In addition, they have been found to be of commercial value due to its immunomodulatory and antitumor activities (Duan *et al.*, 2003; Thude *et al.*, 2006; Classen *et al.*, 2006), as antimicrobial agents (Singha *et al.*, 2003, Kottakis *et al.*, 2007), as emulsifier and as adhesive (Whistler, 1993). Medicinal plants having immune enhancing property such as *Baptisia tinctoria* (Egert and Beuscher, 1992), *Thuja occidentalis*, *Angelica acutiloba* (Kiyohara *et al.*, 1987), *Curcuma longa* (Gonda *et al.*, 1993) also contain AGPs. It has been reported that active constituent of important medicinal plants preparation has been found to be AGP (Classen *et al.*, 2000). The active constituent of the most potent, commonly used immunomodulator from *Echinacea purpurea* has also been found to be AGP (Alban *et al.*, 2002). There are a number of reviews highlighting structure and functions of AGPs in plant growth and development (Majewska and Nothnagel, 2000; Gaspar *et al.*, 2001; Showalter,

2001; van Hengel *et al.*, 2004). Due to its polymorphic nature variations have been found in structure, functions and therapeutic potential of AGPs depending upon it source. Thus to get further information about their structure, functions and to get new potential AGPs of therapeutic importance, there is always a need to explore the AGPs from new sources. In present work efforts are being made to isolate AGP from groundnut and to explore its possible functions and applications.

Groundnut (*Arachis hypogaea* L.) is one of the major oilseed crops and is a member of genus *Arachis* and family Leguminoceae. *A. hypogaea* is an alleotetrapoloid (2n = 4x = 40) species native to South America (Sharma and Bhatnagar-Mathur, 2006). Two major producers in Asia are India with 8.2 million hectare and China with 4.6 million hectare which constitutes 55.9% and 31.6% area of Asia, respectively (Swamy *et al.*, 2003). The seeds of groundnut contain 44-56% oil and 22-30% protein on dry seed weight basis. Oleic and Linoleic are two major fatty acids together account for 75-80% of the total fatty acids in groundnut. Groundnut is an important commodity in many developing countries for both direct human food and oil production, particularly in India where the nitrogen (N)-rich crop residues are also used as fodder. In addition, it is a good source of minerals like P, Ca, Mg and K. Seeds also contains vitamins E, K and B group (Dwivedi *et al.*, 1996).

The production of groundnut in India needs to be increased from the current 8 million tones to about 14 million tones by 2020 to meet the increasing demand of the oil and confectionery industry (Girdhar, 2004). However, the production of groundnut is affected greatly due to various abiotic stresses such as salt, drought and diseases like tikka disease of groundnut, groundnut rosette disease, late leaf spot and rust disease. Thus to meet the high demand of groundnut there is need for development of abiotic stress tolerant and disease free varieties. One of the strategies to achieve this

would be to identify the biomarkers which may be helpful in development of salt stress tolerance and disease free crops. AGP could be one of the potential molecules due to two main facts. Firstly, AGPs have found to induce somatic embryogenesis (Chapman *et al.*, 2000; van Hengel *et al.*, 2001) and secondly AGPs have also been reported to have association with salt stress (Lamport *et al.*, 2006). Although AGPs have been identified as important molecules from several economically important plants, no study regarding these has been carried out in *A. hypogaea* which is one of the most important crops of India. As well variations have been reported in AGPs functions and its therapeutic applications depending upon it source. Therefore, in present study an attempt is being made to isolate the AGP from groundnut (*A. hypogaea)* seedling and to explore its possible cell functions and therapeutic potential. The main objectives of the present work are:

1. Isolation, purification and biochemical characterization of AGP.
2. Generation of polyclonal antibodies against purified AGP and its immunolocalization study.
3. Expression of purified AGP under salt and other abiotic stresses.
4. Study the role of purified AGP in somatic embryogenesis induction.
5. Evaluation of antimicrobial potential of purified AGP.
6. Role as carrier in drug formulation.

# CHAPTER-2

# LITERATURE REVIEW

## 2.1 Introduction

Due to its diverse role in cell growth and development as well as its therapeutic applications, AGPs have been one of the major topics of research for quite some time. Attempts were made to isolate AGPs from several important plants and reveal its structure and possible functions. There are evidences that AGPs are involved in cell-to-cell signaling and extracellular matrix (ECM) interactions during cell differentiation, differentiation of specific tissue or cell type (Majewska and Nothnagel, 2000), cell expansion (Willats and Knox, 1996; van Hengel and Roberts, 2002; Park *et al.*, 2003; Lee *et al.*, 2005), tissue development, sexual reproduction (Sun *et al.*, 2004; Qin and Zhao 2006; Coimbra *et al.*, 2007), somatic embryogenesis (Knox, 1995; Letarte *et al.*, 2006; Tang *et al.*, 2006), cell proliferation (Serpe and Nothnagel, 1994; Langan and Nothnagel, 1997), formation of floral organs (Acosta-Garcia and Vielle-Calzada, 2004; Wiśniewska and Majewska-Sawka, 2006) and root growth (Ding and Zhu, 1997). However, it has been a relatively difficult task to isolate AGPs due to two main reasons. Firstly, AGPs preparations are often heterogeneous in both carbohydrate and polypeptide components and secondly AGP requires extensive deglycosylation prior to sequencing. Therefore, in spite of being an important molecule not much progress could be made for quite some time regarding precise knowledge and possible functions until the effective methods for AGPs purification were developed. However, the development of specific methods for AGP purification, deglycosylation and monoclonal antibodies against AGPs facilitated the study on AGPs. Significant progress has been made in recent past in elucidation of AGPs structure and functions in various plants.

## 2.2 Methods of Purification and Quantification of AGPs

Keeping the importance of AGPs, attempts were made to develop effective methods for AGP purification and deglycosylation. Finally, a specific method for purification of homogeneous AGP was developed. AGPs are differentiated and purified on the basis of their specific affinity for $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside, commonly known as Yariv reagent. Yariv reagent chemically named as 1,3,5-tri-(p-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene, is a synthetic chromophoric molecule (Yariv *et al.*,1962) that specifically binds and precipitates AGPs and used for AGPs purification and detection. (Nothnagel, 1997; Tang *et al.*, 2006; Classen 2007; Wisniewska and Majewska-Sawka, 2006). The Yariv reagent is available commercially (Biosupplies Australia Pvt Ltd, Australia) or can be synthesized easily in the laboratory as per method described (Yariv *et al.*, 1962). Yariv reagent is also used for quantitative estimation of AGPs. There are different methods by which AGPs could be quantified. The first method is to measure the amount of isolated AGP (Bacic *et al.*, 1987). This method requires milligram quantity of purified AGP and is not suitable for routine assay. The second method involves precipitation of AGP by β-glucosyl-Yariv reagent followed by dissociation of the precipitate and colorimetric measurement of the solubilized dye. This method requires a minimum of 15 μg AGP in the sample and 0.5 mg of the β-glucosyl-Yariv reagent for each assay and can not be applied for crude extract or large number of samples. The third method is gel diffusion assay (van Holst and Clarke, 1985), this is also based on the specific interactions of AGPs with β-glucosyl-Yariv reagent. But this method is convenient to perform and it is more sensitive. Sub-microgram quantity of AGP can be quantified using microgram quantity of the β-glucosyl-Yariv reagent. Therefore, this method is more commonly used for AGP quantification.

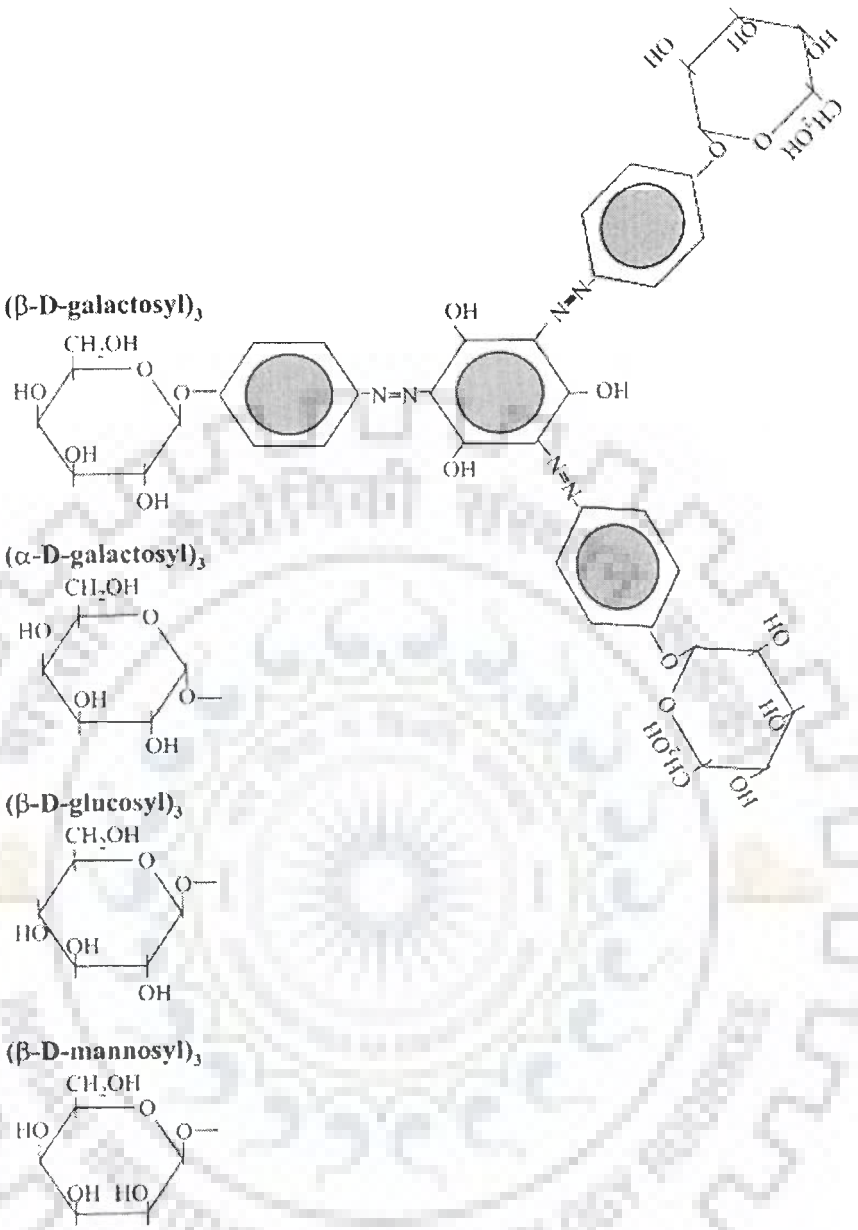



**Figure - 2.1** The chemical structure of Yariv reagent. Yariv reagent can be used to stain AGPs a reddish-brown color in plant sections and to bind and precipitate AGPs for quantitation or purification. The three terminal sugars on this reagent are critical for AGP binding. Certain sugars in these positions allow for AGP binding, whereas other sugars do not and serve as important control reagents. For example, $(\beta\text{-D-galactosyl})_3$ and $(\beta\text{-D-glucosyl})_3$ Yariv reagents bind AGPs, whereas $(\alpha\text{-D-galactosyl})_3$ and $(\beta\text{-D-mannosyl})_3$ Yariv reagents do not bind AGPs. Note that $(\beta\text{-D-galactosyl})_3$Yariv reagent is depicted here with its three (β-D-galactosyl) arms; the names and terminal sugar structures of other commonly used Yariv reagents also are indicated. (Source - Showalter AM (2001) Arabinogalactan-proteins: structure, expression and function. Cell Mol Life Sci **58**: 1399–1417).

## 2.3 Structure of AGP

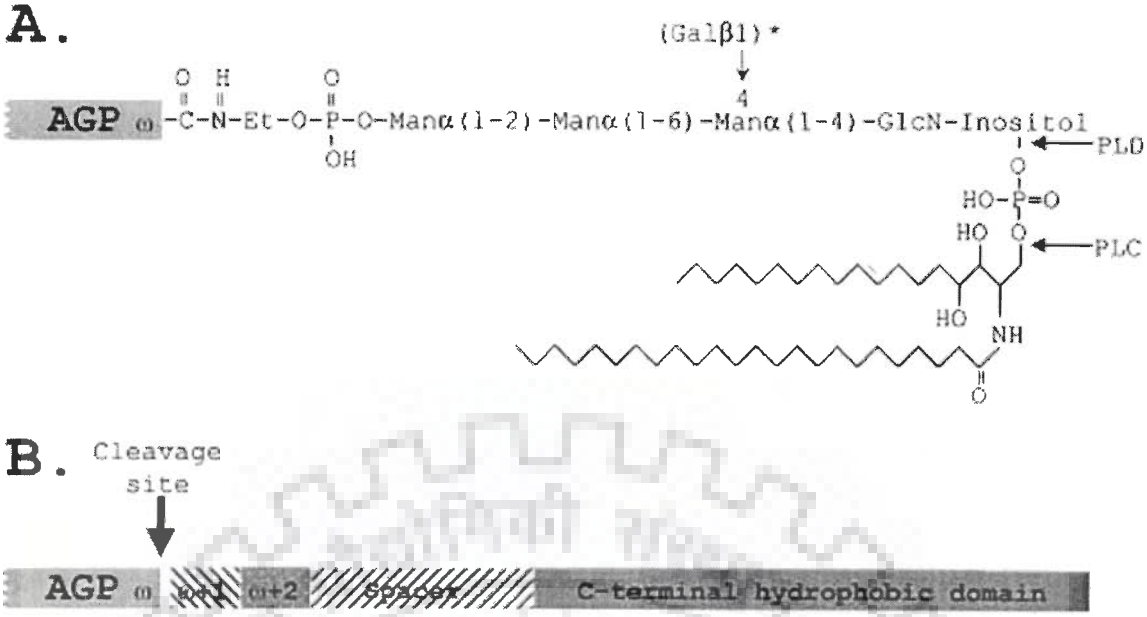
### 2.3.1 Protein component

As adequate purification and deglycosylation procedures were developed, the amino acids composition and amino acids sequence analysis of respective core proteins from carrot (Jermyn and Guthrie, 1985), ryegrass (Gleeson *et al.*, 1989), rose (Komalavilas *et al.*, 1991), maize (Kieliszewski *et al.*, 1992), *Arabidopsis* (Sun *et al.*, 2005) and *Physcomitrella* moss (Lee *et al.*, 2005) gradually appeared in the literature. In general, the polypeptide portion of soluble AGPs is typically rich in Hyp, Ala, Ser, Thr, and Gly residues, although variations have been reported. Hyp-deficient AGPs (Baldwin, 1993; Mollard and Josleau, 1994), His-rich AGP (Kieliszewski *et al.*, 1992) and Ala-poor AGP (Qi *et al.*, 1991) have been found. It is interesting to note that AGP core protein shows extensive sequence divergence within a species, although at least one pair of orthologous AGP genes apparently do exist in tomato (LeAGP1) and tobacco (NaAGP4) which show a high degree of sequence similarity (Showalter, 2001).

In recent past, molecular cloning of several confirmed and putative AGP core proteins has greatly increased our understanding of their structure, post-translational modifications and diversity. Using amino acid sequence and deduced molecular probes, several cDNAs encoding core polypeptides of confirmed AGPs have been isolated and characterized from *Arabidopsis* and other plants (Sommer-Knudsen *et al.*, 1997; Serpe and Nothnagel, 1999; Schultz *et al.*, 2000; Gaspar *et al.*, 2004). Two cDNAs named AGPPc1 and AGPPc2 encode core polypeptides of AGPs secreted in the culture medium of pear cells (Chen *et al.*, 1994; Mau *et al.*, 1995). Three others cDNAs namely AGPNa1, AGPNa2 and AGPNa3 have been isolated from *Nicotiana alata*, while AGPNa1 and AGPNa3 encode core polypeptides of AGPs have been isolated from *N. alata* styles (Du *et al.*, 1996b), the AGPNa2

encodes the core polypeptide of an AGP isolated from the culture medium of *N. alata* cells (Mau *et al.*, 1995). AGP18 (Acosta-Garcia and Vielle-Calzada, 2004), AGP6 and AGP11 encode classical AGP in *Arabidopsis* (Pereira *et al.*, 2006). SOS5 encodes AGP like protein which play role in plant reproduction (Shi *et al.*, 2003).

Based on the deduced protein backbone sequence, AGPs are classified as classical and non-classical AGPs (Bacic *et al.*, 1996; Du *et al.*, 1996a). The classical AGPs have N terminus secretion signal, a central proline/hydroxyproline, alanine, serine and threonine rich region of variable length and a short C- terminus hydrophobic region. The non-classical AGPs do not share a common domain structure, but all classical AGPs have an N-terminus secretion signal, a proline/hydroxyproline, alanine, serine, threonine rich region and a variable, but hydrophobic C-terminal region. In the mature AGP, however, this hydrophobic domain is absent and replaced by a glycosyl phosphatidyl inositol (GPI) lipid anchor (Schultz *et al.*, 1998). The site of this processing is yet to be elucidated in the plant cell but known to occur in endoplasmic reticulum in animal and yeast cell. Amino acid sequence motifs appropriate for such processing have been identified in all known and putative classical AGPs (Borner *et al.*, 2003). Some classical AGPs contain a short domain rich in basic amino acids which interrupt the Pro/Hyp, Ala, Ser and Thr rich domain (Gao *et al.*, 1999). Non-classical AGPs contain either a Cys rich C-terminus or one or two Asn rich domain that enclose the Pro/Hyp, Ala, Ser and Thr-rich domain. None of the known non-classical AGPs contain a hydrophobic C-terminal domain or code for GPI modification. During the last few years GPI-anchored AGPs and its genes have been reported from several plants (Youl *et al.*, 1988; Schultz *et al.*, 1998; Oxley and Bacic, 1999; Svetek *et al.*, 1999; Acosta-Garcia and Vielle-Calzada, 2004; Pereira *et al.*, 2006; Huang *et al.*, 2007).




**Fiugure-2.2** GPI anchor structure and the consensus sequence for its addition. (*A*) Structure of the GPI anchor found in the pear AGP, AGP*Pc*1. The GPI anchor has a partial *b*-galactosyl substitution (*) of its core oligosaccharide and includes a phosphoceramide lipid composed primarily of phytosphingosine and tetracosanoic acid. Potential sites of cleavage by phospholipase C (PLC) and phospholipase D (PLD) are also indicated; cleavage at one or both of these sites would release the AGP from the plasma membrane into the extracellular matrix. (*B*) Consensus sequence present in the C-terminal portion of classical AGPs for the addition of a GPI anchor in the AGP core protein. The amino acid residue designated *w*is the site of GPI anchor addition, while the remaining C-terminal residues are removed during anchor addition. Generally, the *w*residue is Ser, Asn, Ala, Gly, Asp, or Cys, whereas the *w*+ 2 residue is Ala, Gly, Thr, or Ser. The *w*+1 residue is less critical. A 4–8 amino acid spacer region follows which often contains a basic residue (e. g. Arg or Lys) just before the terminal 14–18-amino acid hydrophobic tail. (Source - Showalter AM (2001) Arabinogalactan-proteins: structure, expression and function. Cell Mol Life Sci **58**: 1399–1417).

The precise role that GPI anchors play in functions of AGPs remains unknown. However, its possible role similar to animals in signal transduction is implicated most probably through interaction with other membrane bound molecules (Lease *et al.*, 1998; Lamport *et al.*, 2006). Another possible mechanism, by which GPI-anchored AGPs may be involved in signal transduction, is the phospholipase-mediated cleavage of the protein from its lipid anchor (Knox, 2006).

### 2.3.2 Carbohydrate component

As their name implies, AGPs are rich in arabinose and galactose and in some cases glucuronic acid, along with other less-abundant sugars. The arabinose and galactose residues are arranged in polysaccharide units that are attached to multiple sites on the core protein. The molecular shape of AGP is likely to be influenced by the size of the polysaccharide units attached to the polypeptide core. The size of these polysaccharides varies among different AGPs in the range of 30 to 150 sugars residues and has a (1–3)-β-D-galactan backbone having (1–6)-β-D-galactan side chains, which in turn are modified by arabinose and other less-abundant sugars, including L-rhamnose, D-mannose, D-xylose, D-glucose, L-fucose, D-glucosamine, D-glucouronic acid and D-galacturonic acid (Nothnagel, 1997; Classen 2007). Most AGPs seem to contain a pure (1-3)-β-D-galactan backbone, with exceptions to AGP of *Lolium multiflorum* (Bacic *et al.*, 1987) *and Acacia robusta* (Churms and Stephen, 1984). The (1-3)-α-D-galactan backbone in these are interrupted by other residues. Some AGP molecules have been shown to contain glycans of more than one size. Analysis of AGPs from gum arabic (Qi *et al.*, 1991), *Zea mays* (Kieliszewski *et al.*, 1992), *N. alata* (Schulz *et al.*, 1997) and *Phleum pretense* (Brecker *et al.*, 2005) revealed the presence of large glycan chains and also smaller arabinose oligosaccharides similar to those of extensins. The polysaccharide chains appear to be attached to Hyp residues and possibly to Ser and Thr residues, whereas the short arabinose oligosaccharides, if present, are attached to Hyp. Evidence for galactosyl-*O*-Hyp, galactosyl-*O*-Ser, and arabinosyl-*O*-Hyp and unidentified glycosyl-residues linked to Thr residues are reported for several AGPs (Tan *et al.*, 2003). Based on the Hyp-contiguity hypothesis, which briefly states that contiguous Hyp residues are glycosylated with oligoarabinosides, whereas single noncontiguous Hyp residues are

glycosylated with polysaccharide units, glycosylation patterns can be predicted for AGP core protein sequences (Kieliszewski and Lamport, 1994).

Characterization of the structural details of the carbohydrate chains of AGPs may ultimately proved to be a key to understand the AGPs functions. While functions are primarily associated with the polypeptide portion of many glycoproteins, the situation may be different for AGPs as they consist of high amount of carbohydrate. It remains unclear whether the functionality of AGPs resides in the glycan, the protein, the GPI-anchor or some combination of these. Analyses of AGPs by nuclear magnetic resonance (NMR) suggest that the core polypeptide is locked in position towards the interior of the AGP (Gane *et al.*, 1995). Thus, functional interaction with other macromolecules is sterically much more possible for the peripheral glycosyl residues than for the internalized aminoacyl residues of AGPs. As described in the later sections in this review, the expression studies of AGPs in plants using monoclonal antibodies also confirm the possible functional importance of peripheral glycosyl residues in AGPs, since the epitopes of most of the monoclonal antibodies occur among these peripheral residues (Knox, 1997; Nothnagel, 1997). Thus, variations in terminal glycosyl sequences or other subtle structural features of AGPs may be involved in cell-to-cell communications and cell identity.

## 2.4 Functional Significance of AGPs

### 2.4.1 Functions of AGP in plant growth and development

#### 2.4.1.1 Role of AGPs in cell expansion

Results of several investigations have suggested that AGPs are involved in cell expansion. One such investigation was focused on tip growth in pollen tubes. Growth of lily pollen tubes was reversibly blocked by perturbation of AGPs with $(\beta\text{-D-Glc})_3$ yariv reagent, while growth was not affected by $(\beta\text{-D-Man})_3$ yariv reagent (Jauh and Lord 1996). An *Arabidopsis* mutant isolated and characterized by Takahashi *et al.*,

(1995) exhibited reduced cell elongation leading to short hypocotyls, petioles, stems and roots. Referenced to total detergent-soluble protein, the AGP content of hypocotyls from the mutant was less than wild-type plants. Elongation of suspension-cultured carrot cells, normally inducible by dilution of the culture into medium without 2,4-D, was blocked by 30 mM $(\beta\text{-D-Glc})_3$ yariv reagent but not by $(\alpha\text{-D-Gal})_3$ (Willats and Knox, 1996). The binding of β-glucosyl Yariv reagent with AGP protein has been reported to cause lack of proper cell wall assembly (Roy *et al.*, 1998). Reversible effect of β-glucosyl yariv reagent was observed in *Lilium longiflorum* and *A. cherimola* where β-glucosyl yariv reagent was ceasing pollen tube extension within 1–2 h of treatment, but its removal from the medium allows regeneration of new tip growth in both species (Mollet *et al.*, 2002). Studies on *mur1* mutated *A. thaliana* roots revealed that fucosylated AGPs are essential for cell elongation in roots of *Arabidopsis* (van Hengel and Roberts, 2002). Role of *Physcomitrella patens* AGP in apical cell extension was demonstrated by Lee *et al.*, (2005). They have found localized movement of AGPs from the plasma membrane to the cell wall as a part of the mechanism of tip growth. When AGP1 gene encoding a classical AGP was knocked out, it results in reduced cell length.

**2.4.1.2 Interaction of AGPs with cell wall and plasmalemma**

Because of AGPs presence on outer side of plasmamembrane (Showalter, 2001), another work assigned to AGPs could be interaction with cell wall components. Release of AGPs from the cells between cellulose fibrils can cause separation of microfibrils and thus decrease the rigidity of the cell walls (Schopfer, 1990). Carrot AGPs were reported to interact with cell wall pectins (Carpita and Gibeaut 1993; Baldwin *et al.*, 1993). Asparagine rich domains of non-classical AGPs like AGPNa2 and AGPPc2 can promote the transverse binding of proteins of cell wall

(Mau *et al.*, 1995). AGPs have been reported to play important role in adhesion between the cell wall and plasmalemma (Zhao *et al.*, 2002). Role of AGPs in sperm-egg cell recognition by binding JIM8 and JIM13 on both the plasma membrane of sperm and vegetative cells have been elucidated (Southworth and Kwiatkowski, 1996). AGPs are playing different role in sperm-egg recognition and sperm-central cell recognition during the double fertilization of angiosperms because they have obtained differential expression of the JIM8 epitope in the paired sperm cells, egg cell and central cell of oilseed rape (Pennell *et al.*, 1991). AGPs could be involved in adhesion of some endomembranes (Samaj *et al.*, 1998). In maize roots, AGPs were found in dense contacts of sieve like element reticulum, plasma membrane, plastids and mitochondria. AGPs present in stigma exudates, style-transmitting tissues and pollen could provide recognition signals, nutritional supply, adhesives, lubricants and directional guidance for the pollen tube (Wu *et al.*, 2001). Binding of β-glc yariv to AGPs has been reported to cause inhibition of cell-wall synthesis needed for protonemata differentiation in *Marchantia polymorpha* (Shibaya *et al.*, 2005). Cell surface AGPs of *A. thaliana* are reported to alter the organization of cortical microtubules in the roots. Addition of β-D-glucosyl yariv reagent was found to increase distance between cortical microtubules and plasma membrane (Nguema-Ona *et al.*, 2007).

**2.4.1.3 Role of AGPs in cell differentiation**

AGPs are involved in cell differentiation and plant morphogenesis. Evidences for this function of AGPs are coming from differential expression of AGP at different development stages. Expression of MAC 207 epitopes were strictly related with flower differentiation. The MAC207 epitope was found only in vegetative but not in reproductive cells (Pennell and Roberts, 1990). Developmental regulation of AGPs by

using JIM13 was observed by Knox *et al.*, (1991). They observed that JIM13 was un-reactive with root meristem cells but reacted with developing epidermis, root cap and pre-xylem tissues. AGP epitopes were showing spatial regulation of expression in the pistil tissues of lily (Jauh and Lord, 1996). Involvement of AGPs in differentiation could also be explained by interaction of AGPs and plant hormones (Takos *et al.*, 2000). Ethylene controlled the expression of *AGP2* of *Arabidopsis* (Hall *et al.*, 1999).

AGPs were isolated from styles of *N. alata* and have been compared with tomato LeAGP1 by Gilson *et al.*, (2001). These two AGPs share 78% sequence homology, expressed in the same tissues, at similar levels and both are suppressed by tissue wounding and by pathogen infection. AGP is essential for the differentiation of tracheary elements in *Zinnia elegans* and *Arabidopsis* (Motose *et al.*, 2004). AGPs are involved in the growth and differentiation of maize haploid embryos (Borderies *et al.*, 2004). The importance of AGP in cell differentiation in *Brassica napus* was explained by Tang *et al.*, (2006). They have reported that because of AGP-yariv reagent interaction basic pattern of differentiation has greatly altered at both the radial and apical–basal axes. Pereira *et al.*, (2006) have suggested the role of AGP protein in pollen growth and isolated pollen specific transcripts of AGP6 and AGP11 from *A. thaliana*. Classical lysine-rich AGPs, AtAGP17, 18 and 19 were isolated from *Arabidopsis* (Yang and Showalter, 2007). High AtAGP18 promoter activity was closely associated with vascular tissues, in young organs as well as in styles. Expression level of AtAGP18 was high in roots and flowers.

**2.4.1.4 Role of AGPs in cell division**

It is well established that AGPs are involved in the regulation of cell division in plants. The addition of yariv reagent to rose suspension culture inhibited the culture growth, this effect was concentration dependent and complete growth inhibition was

observed at 50 μM yariv reagent (Serpe and Nothnagel, 1994). Because the cell size was same in the control and test condition, the inhibition of the culture growth was suggested to be associated with suppression of cell division. After being held fully inhibited for 7 days in $(\beta\text{-D-Glc})_3$ yariv reagent containing medium, the cells were transferred to normal medium and there upon resumed growth with a time-course similar to that of control cells. When $(\beta\text{-DMan})_3$ and $(\alpha\text{-D-Gal})_3$ yariv phenylglycosides were used, did not inhibit growth because they can not bind to AGP.

**2.4.1.5 Role of AGP in growth**

In many of the experiments, Yariv reagents have been used as a probe to understand the function of AGP protein in regulation of plant growth. Treatment with Yariv reagent has been found to inhibit growth of cell cultures (Serpe and Nothnagel, 1994), pollen tubes (Roy *et al.*, 1998) and seedlings (Willats and Knox, 1996). Increase in AGP like TTS protein content in the culture medium from 0 to 2 μg/ml has accelerated the growth of *N. tobacum* pollen tubes from 50 to 150 μm/h (Cheung *et al.*, 1995). Seedlings of *A. thaliana* when grown over β-glucosyl yariv reagent containing media they were showing reduced root and shoot growth. The reduced growth was the consequence of reduction in cell elongation during the post-proliferation phase of elongation at the root apex (Willats and Knox, 1996). AGP epitopes were detected from the surface of root hairs of maize seedlings during their development (Samaj *et al.*, 1999). AGP epitopes were found to be relatively higher in amount on root hair initiation sites and on tips of the growing root hairs. Studies on the *reb1* mutation, which was causing disorders in the initial stage of development of many trichoblasts of *Arabidopsis*, emphasize the role of AGPs in the growth of root hairs (Andeme-Onzighi *et al.*, 2002). Because of *reb-1* mutation, there was lack of growth of most root hairs and mutated trichoblasts were not containing specific AGP

epitopes. AGPs are reported to be involved in gibberellin plant hormone function for playing role in plant growth and development and in induction of α-amylase production at plasma membrane of barley aleurone cells (Suzuki *et al.*, 2002). Non-classical AGP gene AtAGP30, which is expressed only in roots of *Arabidopsis*, is required for root germination in *in vitro* condition and seed germination in *in vivo.* The mutant AGP30 was showing abscisic acid induced delay in germination and altered expression of few ABA-regulated genes (van Hengel and Roberts, 2003). AtAGP30 gene encodes an AGP which plays role in root regeneration whose tissue-specific pattern of expression was controlled by abscisic acid (van Hengel *et al.*, 2004). The role of AGPs in cell wall regeneration process was observed when β-glc Yariv reagent was added to protoplasts culture medium of *M. polymorpha* L. during the period of incipient cell wall regeneration which decreases the survival rate of regenerated cells. After the period of incipient cell wall regeneration when β-glc Yariv reagent was added in to media it was not producing any decrease in survival rate (Shibaya and Sugawara, 2007).

**2.4.1.6 Role of AGPs in sexual reproduction**

Pollen-specific AGPs exist in several organisms such as *Arabidopsis* (Pereira *et al.*, 2006), *B. napus* (Gerster *et al.*, 1996) and *Alfalfa* (Qiu *et al.*, 1997) which indicate the important role of AGPs in pollen development. Two pollen-specific putative AGP genes are Sta 39-4 and Sta 39-3 which were isolated from *B. napus* flowers (Gerster *et al.*, 1996). The identity between these two genes is 95% at the nucleic acid level and 98% identical at the amino acid level. Sta 39 transcripts may appear following microspore mitosis at the binucleate stage and accumulate until the pollen is fully mature. Xylem-specific PtaAGP3 was isolated from differentiating xylem of loblolly pine (Loopstra *et al.*, 2000). Five AGPs and AGP like molecule

were isolated from style and stigma of *N. alata* (Sommer-Knudsen *et al.*, 1997). These five molecules were different in their expression. AGPNa1 was expressed in style as well as in other organs also. In cell cultures, expression of AGPNa2 was very low in styles. AGPNa3 expression was observed in pistil only. The proline rich NaPRP4 and NaPRP5 were showing high expression in transmitting tract of style. Function of AGPs in plant sexual reproduction arose from studies with *Nicotiana* plants, in which transmitting tissue-specific AGPs were shown to promote pollen tube growth *in vitro* and to attract pollen tubes in a semi *in vivo* pollen tube growth system (Wu *et al.*, 2000). On basis of immunolabelling studies with monoclonal antibodies MAC207, JIM8 and JIM13 in pistil tissues of *Actinia deliciosa* and *Amaranthus hypochondriacus*, Class III pistil-specific extensin-like proteins (PELPIII) were reported which were important for pollen tube growth and also having characteristics of AGPs. They also can bind with β-glucosyl yariv reagent (Bosch *et al.*, 2001). AGPs are also participating in pollen-pistil interactions (Coimbra and Duarte, 2003). They have proposed that AGP can work as chemoattractants or nutrient suppliers for pollen tube growth. AGP18 is a classical AGP gene that specifically express during female gametophyte development in *Arabidopsis* (Acosta-Garcia and Vielle-Calzada, 2004). They specifically degraded the endogenous AGP18 transcript by RNA interference (RNAi), which results in 75% of the primary transformants lines showing reduced seed set. Genetic and molecular analysis of a line containing a single T-DNA RNAi insertion suggests that post-transcriptional silencing of AGP18 is acting both at the sporophytic and gametophytic levels. The cytological analysis of all defective AGP18-RNAi lines indicates that AGP18 is essential to initiate female gametogenesis in *Arabidopsis*. JIM13 labeling in zygotic embryos of *A. thaliana* showed that AGPs were mainly located in the embryo proper, top 1 to 2 cells and basal part of

suspensors. Addition of β-glc yariv reagent in ovule culture medium of *A. thaliana* has resulted in decreased survival rate and frequency of development of ovules at the zygote stage. Inhibitory effects were reversible in a concentration-dependent and time-dependent manner as yariv reagent was removed from the culture media (Hu *et al.*, 2006). Role of AGP in sexual reproduction was explained by Qin and Zhao (2006). They have found abundant AGPs in unfertilized egg cells, however, the level of AGPs substantially decreases in fertilized egg cells. Coimbra *et al.*, (2007) have performed labelling of arabinogalactan protein with JIM8, JIM13, MAC207 and LM2 monoclonal antibody during *Arabidopsis* pollen and pistil development and suggest that some AGPs can work as markers for gametophytic cell differentiation. BcMF8 (Brassica campestris male fertility 8) gene was isolated from *Brassica campestris* L. which has features of classical AGPs in its deduced amino acid sequence in fertile floral bud but was silent in sterile A/B lines. They have also reported that BcMF8 gene has high sequence identity with known pollen-specific AGP genes Sta 39-4 and Sta 39-3 isolated from *B. napus* (Huang *et al.*, 2007).

**2.4.1.7 Role of AGP protein in somatic embryogenesis**

Establishment of role of AGPs in inducing somatic embryogenesis has involved the amount or types of AGPs in the culture medium. Another important factor is precipitation of AGPs with either $(\beta\text{-D-Glc})_3$ yariv reagent or anti-AGP antibodies (Thompson and Knox, 1998; Butowt *et al.*, 1999), or by adding exogenous AGPs. In certain stages of somatic embryogenesis presence of the JIM4 epitopes supports role of AGPs in embryo growth and differentiation (Stacey *et al.*, 1990). Adding exogenous AGPs has been reported to enhance the efficiency of somatic embryogenesis in carrot and Norway spruce (Kreuger and van Holst, 1996). AGPs from carrot cell line were isolated and have found that pattern of extracted AGPs were

changing according to the developmental stage of cell line (Kreuger and van Holst, 1993). They have added carrot seed AGPs to a non embryogenic, two year old cell line and it was found that AGP was able to induce embryogenic potential in 2 year old cell line. Kreuger and van Holst (1993) and Egertsdotter and von Arnold (1995) have shown that AGPs secreted from culture cells or extracted from seeds affect the induction and development of somatic embryos. Seed AGP isolated from carrot and tomato was analyzed with the help of monoclonal antibody ZUM15 and ZUM18. The ZUM18AGP fraction was able to increase the percentage of embryogenic cells from 40% to 80% in a week time (Kreuger and van Holst, 1995). JIM8 labels a sub-population of cells of developmental path of somatic embryogenesis, these cells are thought to provide a signal essential for embryo formation in carrot cell cultures (McCabe *et al.*, 1997). In presence of β-Glc yariv reagent early stage embryos were not able to progress on to next stage of development (Thompson and Knox, 1998). Yariv reagent was able to block somatic embryogenesis obtained from *Cichorium* hybrid '474' in a concentration dependent manner (Chapman *et al.*, 2000). When yariv reagent treated roots were transferred to control condition they were able to reinduce somatic embryogenesis. AGPs were mainly localized in the protodermal cells of globular somatic embryos in *Euphorbia pulcherrima*. From embryogenic and non-embryogenic callus different sets of AGPs were characterised using yariv reagent and LM2 monoclonal antibody (Saare-Surminski, 2000). Endochitinase sensitive, glucosamine and N-acetyl-D-glucosamine containing AGP protein was isolated from culture of *Daucus carota* (van Hengel *et al.*, 2001). It was observed that AGP protein was able to induce embryogenesis in protoplast. AGP was also able to reinitiate cell division in a non-dividing protoplast. They suggested that AGP molecule may stimulate somatic embryogenesis by establishing cell identity and mechanisms of cell-

to-cell signaling. In immature carrot seeds, AGP was found to increase somatic embryogenesis and its expression was developmentally regulated. After fertilization degradation of maternally derived AGPs were found and new AGPs were formed during cellularization of the endosperm. Potential for induction of somatic embryos of AGPs were also found to be dependent on the age of the seeds (van Hengel *et al.*, 2002). AGP was isolated from maize microspore culture media whose concentration was increasing with time of culture (Borderies *et al.*, 2004). It was observed that AGP can stimulate development of microspore obtained from non responsive genotype. Letarte *et al.*,( 2006) have shown the role of AGP in somatic embryogenesis of wheat microspore culture. They observed that by using AGP from gum arabic, they were able to obtain green plants from wheat microspore culture without the presence of ovaries. AGP when binds β-D-glucosyl yariv reagent disrupt microspore embryogenesis in a concentration-dependent manner in *Brassica napus*. (Tang *et al.*, 2006). A classical high Hyp content AGP was identified from Cashew nut tree exudates gum. They observed that cashew nut gum AGP is not only able to stimulate carrot somatic embryogenesis after 2 and 3 weeks of culture, but also enhance conversion of somatic embryos into plantlets (Pereira-Netto *et al.*, 2007).

**2.4.1.8 Role of AGPs in programmed cell death**

In plants, programmed cell death (PCD) is a normal developmental process involved in anther, megagametophyte and vascular tissue development (Groover and Jones, 1999; Groover *et al.*, 1997) as well as in senescence (OrzaÂez and Granell, 1997), pollination and sex determination (Chasan, 1994; Dietrich *et al.*, 1994). (β-D-galactosyl)$_3$ yariv reagent inhibits the growth of *Arabidopsis* suspension cultured cells by inducing these cells to undergo PCD in a time and dose-dependent manner (Gao and Showalter, 1999). These results implicate that AGPs are involved in PCD in

plants and indicate that AGPs may be important component of the signal transduction pathway for this process. Same results were also observed by Langan and Nothnagel (1997) where they have noted that $(\beta\text{-D-galactosyl})_3$ yariv reagent was able to kill *Arabidopsis* cell cultures. Morphological studies like cell shrinkage, cytoplasmic condensation and DNA ladder obtained on agarose gel electrophoresis revealed that addition of $(\beta\text{-galactosyl})_3$ Yariv reagent induced programmed cell death in tobacco BY-2 suspension cultured cells (Chaves *et al.*, 2002).

### 2.4.2 Therapeutic applications of AGPs

Due to its diverse biological activities and high water solubility AGPs are found to be of great therapeutic value and have been one of the major areas of research during recent two decades. Some of the major therapeutic potentials are highlighted here.

#### 2.4.2.1 Role of AGPs as immunomodulators

Most of the diseases develop directly or indirectly due to impairment of defense system of the body. Modulation of the immune response through stimulation or suppression may help in maintaining a disease free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide safer alternative therapy to conventional chemotherapy. Polysaccharides and glycoconjugates with potent immunostimulatory activity have been isolated from diverse sources (Hauer and Anderer, 1993; Han *et al.*, 1999; Classen *et al.*, 2000; Zhang *et al.*, 2002; Duan *et al.*, 2003; Kim *et al.*, 2003; Classen *et al.*, 2006).

The most potent immunostimulants used now days are obtained from Larix (*Larix occidentalis)* and *Echinacea purpurea.* The polysaccharide and proteoglycans enriched fractions found to be responsible for activity and Arabinogalactan (AG) and

AGPs are part of the active principle (Hauer and Anderer, 1993; Classen *et al.*, 2000; Classen *et al.*, 2006). AGPs have been isolated from roots of *Baptisia tinctoria*, *E. pallida* and *E. purpurea* (Classen *et al.*, 2006). They have evaluated the immunological potential of isolated AGP for proliferation and production of IgM lymphocytes, nitrite and IL6-production of mouse macrophages. The AGP isolated from *B. tinctoria* and *E. pallida* was showing promising results. AGPs interactions with the complement system have been demonstrated *in vitro* (Alban *et al.*, 2002), and binding of an AGP from *E. purpurea* to human lymphocyte, monocytes and granulocytes has been proven by fluorescent activated cell sorter (FACS) analyses (Thude *et al.*, 2005). They have explained that binding of AGP to these cells is not via CD4 and CD8 receptor. AGP protein isolated from *Andrographis paniculata* reduces hepatic renal alcohol toxicity, if given at 125 mg/kg body weight of mice. This reduced toxicity was comparable to hepatoprotactive agent silymarin (Singha *et al.*, 2007). It has been suggested that arabinogalactan is an attractive potential drug carrier due to its high water solubility (>70% in water). The coupling of amphotericin-B (AmB), a water-insoluble antifungal and antileishmanial agent, to arabinogalactan (AG) shown that AG–AmB conjugate was soluble in water and exhibited improved stability in aqueous solutions as compared to the unbound drug. The conjugates showed comparable inhibitory concentration values against the pathogenic yeast *Candida albicans*, and against *Leishmania major* parasites. In some cases the arabinogalactan component alone has been observed to execute immune enhancing property. Chintalwar *et al.*, (1999) have isolated immunologically important arabinogalactan from stem of *Tinospora cordifolia*. Arabinogalactan DL-3Bb, isolated from the leaves of *Diospyros kaki* has been observed to stimulate lipopolysaccharide (LPS)-induced B lymphocyte proliferation (Duan *et al.*, 2003). Arabinogalactan

isolated from *Phleum pretense* was showing IgG4 reactivity (Brecker *et al.*, 2005). Arabinogalactan isolated from *Vernonia kotschyana Sch.* has been reported to show complement fixation activity and a T cell independent induction of B-cell proliferation. Arabinogalactan was also able to induce chemotaxis of human macrophages, T cells and NK cells (Nergard *et al.*, 2005).

Thus it is concluded that AGPs and like molecules are a natural plant derived glycoconjugates of great therapeutic potential. They are widely distributed throughout the plant kingdom from lower to higher plants. They are highly polymorphic molecule and its therapeutic potential depends on source. Since India has vast resource of diverse class of valuable medicinal plants which remain unexplored, attempts should be made to explore this valuable resource as some of these could be a rich and potent source of such therapeutically important glycoconjugates.

#### 2.4.2.2 Antimicrobial property of AGP

Not much work has been done about the antimicrobial property of AGP, but in recent past antifungal and antibacterial activities of AGP have been observed by Singha *et al.*, (2003). Kottakis *et al.*, (2007) also have showed that AGP isolated from *Pistacia lentiscus* was having antibacterial activity against *Helicobacter pylori*.

### 2.4.3 Other miscellaneous applications of AGPs

AGPs are important constituents of extracellular secretions. Root slimes of wheat, cowpea and maize contain (1-3), (1-6) and (1-3-6 linked) galactopyranosyl and Hyp residues and are able to bind with yariv reagent. AGP contribute in the protective and lubricative properties of slime (Moody *et al.*, 1988). The best known gum, containing AGP is gum arabic (Stephen, 1983; Whistler, 1993; Osman *et al.*, 1995). AGP containing gum arabic is a known emulsifier and have low viscosity even at

higher concentration. Gum obtained from *Acacia seyal* and *Acacia lactia* also contain AGP (DePinto *et al.*, 1998; Gammon *et al.*, 1986). Plants from fabaceae (Anderson *et al.*, 1990), Cactacea (Goycoolea *et al.*, 1997) also contain AGP. AGP can act as adhesive that prevent pathogen entry and desiccation by sealing wounded tissues. Pereira-Netto *et al.*, (2007) have isolated a classical AGP from cashew-nut tree exudates gum (CNTG), which was stimulating somatic embryogenesis.

# CHAPTER-3

# MATERIALS, METHODS, RESULTS & DISCUSSION

# PART-A

# ISOLATION, PURIFICATION & IMMUNOLOCALIZATION OF AGP

## 3.1 Introduction

AGPs are widely distributed among the plant kingdom from bryophytes to angiosperms and they probably occur in many different plants and in many different parts of plants, on the plasma membrane, on the cell wall, extracellular matrix and plant exudates. AGPs are implicated in many aspects of plant growth and development including cell proliferation, expansion, cell signaling and cell apoptosis. Besides, AGPs and like molecules are also found to have potential application as immunomodulators, emulsifiers, adhesives, active ingredient of drug formulations, several herbal medicines and nutritional preparations, which are of great biotechnological and commercial significance. However, it has been observed that AGPs are highly polymorphic molecules and variations are found in structure and functions of these molecules depending upon it source. Because of its polymorphic nature it has been a difficult task to establish the precise functions of AGP molecules. Although success has been achieved but efforts are still on to understand its full functions. It has also been observed that the therapeutic potential of the AGPs differ from source to source. Therefore, it is highly desirable to isolate, purify AGPs from difference sources and explore its possible cellular functions and evaluate their possible therapeutic applications. Keeping the above facts in mind, attempts were made in the present work to isolate and purify AGP from groundnut and to explore some of its cellular and therapeutic potential. The present section describes the

strategies and methodologies used for isolation, purification and biochemical characterization of AGP.

## 3.2 Materials and Methods

### 3.2.1 Materials

All chemicals and solvents used in the present study were obtained from M/s Sigma-Aldrich (USA), Pierce Chemicals (USA), Merck India, BDH (India) and SRL (India). Specific chemicals were purchased from their respective sources such as Freund's adjuvant from Sigma-Aldrich (USA) and goat anti rabbit IgG-HRP conjugate from Santa Cruz Biotech. Inc. (USA). Large variety seeds of groundnut were obtained from the National Seed Centre, Roorkee, Uttarakhand, India.

### 3.2.2 Pretreatment of seeds and growth conditions

Sterilization of seeds was done as per method described (Rohini and Rao, 2000). Seeds were surface sterilized by treatment with disinfectant 0.1% $HgCl_2$ for 5 min with continuous stirring, washed thoroughly and imbibed for 12 h in sterile water. Imbibed seeds were placed on moist stack of blotting sheets for sprouting. After that, well sprouted seeds with uniformity were selected and transferred to plastic trays for growing hydroponically in Hoagland's medium (Hoagland and Arnon, 1950) under controlled temperature (28 °C) in a plant growth chamber in dark at 80% relative humidity. Medium was changed after every 48 h to avoid nutrient depletion. Seedlings were harvested after seven days for the experimental work.

### 3.2.3 Composition of nutrient solution

Nutrient solution for hydroponics was prepared with the following composition.

**Table 3.1** Ingredients of Hoagland's nutrient medium for groundnut

| Ingredients | Stock Solution | Working Concentration | Volume/Litre added |
|---|---|---|---|
| $KNO_3$ | 10.1 g/100 ml | 5 mM | 5 ml |
| $Ca(NO_3)_2$ | 23.6 g/100 ml | 5 mM | 5 ml |
| $MgSO_4.7H_2O$ | 24.6 g/100 ml | 2 mM | 2 ml |
| $KH_2PO_4$ | 13.6 g/100 ml | 1 mM | 1 ml |
| $H_3BO_3$ | 0.618 g/100 ml | 0.05 mM | 5 ml |
| $MnSO_4.H_2O$ | 0.169 g/100 ml | 9.00 µM | 900 µl |
| $ZnSO_4.7H_2O$ | 0.28 g/100 ml | 0.77 µM | 77 µl |
| $CuSO_4.5H_2O$ | 0.249 g/100 ml | 0.30 µM | 30 µl |
| $Na_2MoO_4.2H_2O$ | 0.241 g/100 ml | 0.10 µM | 10 µl |
| *Fe-EDTA | | 0.05 mM | 5 ml |

* Fe-EDTA stock solution was made by adding 0.27 g of $FeCl_3.6H_2O$ and 0.29 g of EDTA to 100 ml of doubled distilled water. The nutrient solution was autoclaved and then Fe-EDTA solution was added aseptically.

### 3.2.4 Isolation and purification of arabinogalactan proteins from *A. hypogaea*

Seeds of *A. hypogaea* large variety were surface sterilized and washed thrice with ultra pure water. The seeds were soaked in sterilized water for 24 h, and then kept in dark for sprouting. The germinated seeds were further grown in Hogland's medium, in a plant growth chamber under controlled condition of light and temperature. Seven days old seedlings were collected for AGP isolation. The isolation and purification of AGP was carried out as described (Schultz *et al.*, 2000) with slight modification. To extract AGP, 10 g (fresh weight) of seedling tissue were cut into small pieces and grounded to fine powder in liquid nitrogen. To the ground tissues, 10

ml of extraction buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.1% β-mercaptoethanol and 1% (w/v) Triton X-100) was added and incubated at 4 °C for 3 h. Samples were centrifuged for 10 min at 14000×*g*. The supernatant was precipitated with 5 volume of ethanol at 4 °C, overnight. The pellet was resuspended in a 5 ml of 50 mM Tris–HCl pH 8.0. The insoluble material was removed by centrifugation and supernatant was collected. The pellet was resuspended in additional 5 ml of 50 mM Tris–HCl pH 8.0. The supernatant were pooled together and freeze dry overnight to concentrate the sample. The dried sample was dissolved in 500 µl of 1% (w/v) NaCl and transfer to a 1.5 ml microcentrifuge tube. AGPs were precipitated with the β-glucosyl Yariv reagent (β-GlcY) by mixing the resuspended sample with equal volume of β-GlcY (2 mg/ml) in 1% NaCl and incubated at 4 °C, overnight. The insoluble AGP-Yariv complex was collected by centrifugation at 14000×*g* in a microcentrifuge for 1 h. The pellet was washed with 0.1M NaCl and deionized water. Sodium hydrosulphide ($Na_2S_2O_4$) was added to final concentration of 10% (w/v) to decompose β-GlcY. The solution was heated to 50 °C till the red color disappears. The samples were dialyzed extensively against water at 4 °C and freeze dried.

### 3.2.5 Synthesis of β-glucosyl Yariv reagent

Synthesis of β-glucosyl yariv reagent was performed by the method described by Yariv *et al.*, (1962). p-nitrophenyl β-glucoside (Sigma, St. Louis, MO) was reduced at atmospheric pressure of hydrogen over platinum oxide. The p-aminophenyl β-glucoside was re-crystallized from ethanol. Four milli moles of p-aminophenyl β-D-glucoside were diazotised in 24 ml of 0.5 M HCl at 0°C. 4 mM of $NaNO_2$ was added to a solution of 1 mM phloroglucinol in 50 ml water (pH 9.0). The pH was kept stable at 9.0 by adding 0.5 N NaOH. After about 2 h the pH became

stable and an equal volume (150 ml) of methanol was added to precipitate the β-glucosyl Yariv reagent. The precipitate was collected by centrifugation (3000xg, 15 min) and the supernatant was discarded. The precipitate was dissolved in water and re-precipitated. The residue was dried at 50 °C and stored at room temperature.

### 3.2.6 Quantification of AGP

AGP determination was done by glucosyl Yariv reagent binding in a radial gel diffusion assay. Agarose gel (1%) containing 0.02% Yariv reagent, 0.15 M NaCl, 0.02% sodium azide were poured into petri dishes and solidified. Wells were made (4 mm diameter) in the gel using cork borer. 15 µl and 30 µl of AGP solution (4 mg/ml) were loaded in to the wells. Buffer alone without AGP was used as control, and gum arabic and larch AG (Sigma, USA) were used as positive and negative test control, respectively. The petri dishes were sealed with parafilm and kept in dark at room temperature for 2 days to allow the color to develop.

### 3.2.7 Protein and sugar estimation

The concentration of protein in the sample was estimated by Bradford (1976) assay. BSA was used as protein standard. The concentration of sugars in the sample was estimated by the method described by Dubois *et al.*, (1956). Glucose was used as carbohydrate standard.

### 3.2.8 SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) with little modifications.

#### 3.2.8.1 Preparation of reagents

The stock solutions of various gel components were prepared as mentioned below.

1. **Acrylamide solution (30%):** 30% w/v acrylamide solution containing 0.8% w/v N, N-methylene-bis-acrylamide: 29.2 g of acrylamide and 0.8 g of bisacrylamide was dissolved in 70 ml of deionised water. When acrylamide was completely dissolved, water was added to make a final volume of 100 ml. Solution was filtered with a whatman No.1 paper and was stored at 4 °C in a dark bottle.

2. **Resolving buffer (1.5 M Tris-Cl, pH 8.8):** 18.2 g of Tris base was dissolved in 80 ml of water and the pH was adjusted to 8.8 with HCl and water was added to make a final volume of 100 ml and was stored at 4 °C.

3. **Stacking buffer (0.5 M Tris-Cl, pH 6.8):** 6.1 g of Tris base was dissolved in 80 ml of water and pH was adjusted to 6.8 with HCl and water was added to make a final volume of 100 ml and was stored at 4 °C.

4. **Sodium dodecyl sulphate (SDS, 10% w/v):** 10 g SDS dissolved in 60 ml of water and kept at room temperature overnight without shaking. Then the volume was leveled at 100 ml by distilled water and was stored at room temperature.

5. **Catalyst: 10% ammonium per sulphate (APS):** 10 mg APS was dissolved in 100 µl of water (Freshly prepared solution was used).

6. **TEMED (N, N, N, N-tetra methylethylenediamine):** It was used undiluted from the bottle stored at cool, dry and dark place.

7. **Electrode buffer:** (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3) 0.3 g Tris base, 1.4 g glycine, 1 ml 10% SDS/100 ml electrode buffer. Electrode buffer was also prepared as stock solution 5X concentration, consisting of 15 g Tris base, 72 g glycine and 5 g SDS/litre. It was stored at

room temperature and was diluted to 5 times by adding 4 parts of distilled water before use.

8. **Stock sample buffer (2X)** (0.125 M Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.002% Bromophenol blue, 10% β-mercaptoethanol).

The sample buffer (2X) was prepared by mixing the stock solutions as per given composition.

**3.2.8.2 Preparation of sample buffer**

| Ingredient | Volume |
|---|---|
| Water | 3.0 ml |
| 0.5M Tris-Cl, pH 6.8 | 2.5 ml |
| 10% SDS | 2.5 ml |
| Glycerol | 2.0 ml |
| 0.5% Bromophenol blue (w/v) | 0.5 mg |

Stored at room temperature, SDS-reducing sample buffer was prepared by adding 100 µl of β-mercaptoethanol to each 0.9 ml of stock sample buffer, before use.

**3.2.8.3 Casting of gel**

Ten percent denaturing discontinuous gel was prepared by mixing gel stock solutions as per given composition. The monomer solution was prepared for resolving gel by mixing all of the reagents given below except the ammonium per sulfate and TEMED.

**Recipe for resolving gel (10%): (10 ml)**

| Ingredient | Volume |
|---|---|
| Acrylamide solution 30% | 3.3 ml |
| 1.5 M Tris-Cl pH 8.8 | 2.5 ml |
| 10% SDS | 0.1 ml |
| 10% APS | 0.1 ml |
| Water | 4.0 ml |
| TEMED | 5.0 µl |

**Recipe for stacking gel (5%): (5 ml)**

| Ingredient | Volume |
|---|---|
| Acrylamide solution 30% | 0.83 ml |
| 0.5 M Tris-Cl pH 6.8 | 0.63 ml |
| 10% SDS | 0.05 ml |
| 10% APS | 0.05 ml |
| Water | 3.40 ml |
| TEMED | 5.00 µl |

The solution was degassed for at least 3-5 min. The APS and TEMED were gently mixed into the degassed monomer solution. The solution was well mixed uniformly and poured gently in between the plates. The resolving gel was cast up to 2/3 height on pre marked plates followed by layering of 200 µl butanol overlaying solution. After 15 min, the demarcation occurred between the acrylamide layer and butanol layer indicated the complete polymerization of gel. Butanol was decanted and the space was washed with distilled water. Similarly 5% stacking gel was also layered on top of the resolving gel. The wells were cast in stacking gel by placing the teflon comb in between and at the top of the two plates. After 15-20 min the comb was removed carefully.

**3.2.8.4 Sample preparation**

Protein concentration 150 µg/50 µl were mixed with 1 volume of sample treatment buffer (0.125 M Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.002% bromophenol blue, 10% β-mercaptoethanol) and heated to boiling point for 5 min.

**3.2.8.5 Electrophoresis**

Electrophoretic separation was done by using BIO-RAD Mini-PROTEAN® 3 Cell electrophoresis unit. The prepared samples were loaded into the wells with a protein concentration of 30 µg of each sample and electrophoresed at 80 V through stacking gel. Once the sample was concentrated at the interface of the stacking and

separating gel as sharp blue line, the voltage was increased to 120 V and the electrophoresis was continued until the tracking dye reached at the bottom of the gel.

#### 3.2.8.6 Staining and destaining of gel

After the run, the gel was removed from plates and put in staining solution (0.1% coomassie brilliant blue R-250 w/v in 40% methanol, 10% acetic acid w/w) for 4-6 h with mild shaking at room temperature. Then, the gel was destained with several changes of destaining solution I (40% methanol, 10% glacial acetic acid) and finally kept in destain II (10% glacial acetic acid).

### 3.2.9 Alcian blue staining of gel

Alcian blue staining of gel was performed as described by Wardi and Michos (1972). The polyacrylamide gel was placed in 12.5 % TCA solution for 30 min, rinsed with distilled water and placed in 1% periodic acid (in 3% acetic acid) for 50 min. Excess periodate was removed by placing gel in 0.5% potassium metabisulfite for 30 min. The gel was washed with distilled water and placed in 0.5% alcian blue solution (in 3% acetic acid) for staining. Destaining of gel was done by 7% acetic acid solution. The destained gel was pictured in Bio-RAD Gel Doc XR system.

### 3.2.10 Yariv staining of the gel

Yariv staining of the gels were performed as per method described by Zhu *et al.*, (1993). Gels were stained with 30 µg/ml yariv reagent in 10% (v/v) dimethyl sulfoxide overnight and destained with 2% (w/v) NaC1 overnight and photographed.

### 3.2.11 Production of anti AGP polyclonal antibodies

For production of polyclonal antibodies two male rabbits (approximate 3kg body weight) were procured from All India Institute of Medical Science (AIIMS), New Delhi. Pre-bleeding was done for both the animals, one week before the immunization. An emulsion was prepared by mixing 500 µg of protein with 2 ml of

freund's complete adjuvant and equal volume of PBS pH 7.2. The animals were immunized with this emulsion at various locations by hypodermic syringe. All the injections made were of subcutaneous type. At 14$^{th}$ day, the first booster dose was administered with the same antigen emulsified with freund's incomplete adjuvant. On 28$^{th}$ day, second booster dose was administered in a similar fashion. Third booster dose was given on 42$^{nd}$ day, in a similar way. After four days animals were bled through the marginal ear vein. After bleeding, blood was stored in graduated test tube and incubated for 60 min at 27°C, for clotting. Pale golden color serum was collected from the clotted blood and stored in aliquots of 500 µl, at -80 °C.

### 3.2.12 Enzyme linked immuno sorbant assay (ELISA)

For measurement of antibody titer in post immune serum, Enzyme Linked Immuno Sorbant Assay (ELISA) was used.

#### 3.2.12.1 Reagents

1. Blocking buffer: 3% BSA in PBS
2. Pre serum: Serum collected from the rabbit before the immunization
3. Primary antibody: Anti-serum collected from the rabbit after immunization
4. Secondary Antibody: Commercially available Goat anti rabbit IgG coupled with horseradish peroxidase enzyme (Goat anti rabbit IgG-HRP) was used as secondary antibody.
5. 0.1M Citric acid
6. Phosphate citrate buffer pH 5.0
7. 0.2 M Sodium dihydrogen phosphate
8. Substrate: Ortho phenylene diamine (OPD) 4 mg, 0.1 M Citrate buffer (4.8 ml), 0.2 M Phosphate buffer (5.15 ml) and 30% hydrogen peroxide (4µl)

   The substrate was freshly prepared and $H_2O_2$ was added just before the use.

9. Stopping reagent: 4 N $H_2SO_4$ (4ml): distilled water (32ml)

### 3.2.12.2 Procedure

1. Microtiter plate was coated with 50 µl of immunizing antigen containing 5 µg of protein in each well and incubated overnight at 4 °C.
2. Liquid content of the well was removed and wells were washed three times by 100 µl washing buffer (TBS)
3. After washing 100 µl blocking buffer was added in each well and kept for 90 min at room temperature.
4. The wells were washed by TBS, thrice.
5. 50 µl of different serial dilutions of pre-immune and post-immune sera were taken in well of microtiter plate, in duplicate. The microtiter plate was incubated at room temperature for 90 min.
3. Remove the unbound serum by washing three times with TBS.
4. 50 µl of secondary antibody (Goat anti rabbit IgG-HRP conjugate, 1:1000 dilutions) were added to each well and incubated at 37 °C for 90 min.
5. Wash the plate twice with TBS.
6. 10 ml of Ortho phenylene diamine reagent (4 mg OPD was added in 4.8 ml of 0.1 M citrate buffer and 5.15 ml of 0.2 M phosphate buffer pH-5.0, 4µl of $H_2O_2$ was added just before the use) was added to each well and incubated at room temperature in dark for 20 min.
7. The reaction was stopped by addition of 50 µl of 4N $H_2SO_4$ in to each well.
8. Absorbance was recorded with in 10 min at 492 nm in an ELISA reader (Metertech Inc, model Σ 960).

### 3.2.13 Western blotting

Western blotting was performed as per the method of Towbin *et al.*, (1979) with slight modifications. It comprises a series of steps involving:

1. Resolution of a complex protein sample in a polyacrylamide gel by SDS-PAGE.
2. Transfer of the resolved proteins to a membrane by electrotransfer.
3. Identification of a specific protein on the membrane by antibody binding named as immunoblotting.

#### 3.2.13.1 Electrophoretic separation and transfer of protein bands

The protein sample was resolved on 10% SDS-PAGE as explained earlier. Unstained gel was used for electrophoretic transfer of protein bands to Polyvinylidene Difluoride (PVDF) membrane using wet blotting system in Mini Trans-Blot® Bio-Rad.

#### 3.2.13.2 Reagents

1. Polyacrylamide gel containing the resolved proteins
2. Immobilon PVDF transfer membrane, cut to the same dimensions as the gel (including notched corner for orientation purposes)
3. Two sheets of Whatman® 3MM filter paper or equivalent, cut to the same dimensions as the gel
4. Scotch Brite® pads
5. Tank transfer system large enough to accommodate gel
6. Methanol, 100%
7. Milli-Q® water
8. Tris/glycine transfer buffer: 25 mM Tris base, 192 mM glycine, 10% (v/v) methanol, pH 8.3).
9. Powder free gloves, forceps, glass plates, glass rods etc.

### 3.2.13.3 Assembly of transfer cassette

1. The gel from its glass cassette was removed and stacking gel trimmed away.
2. The gel was immersed in transfer buffer for 15 to 30 min.
3. The filter papers and scotch brite pads were soaked in transfer buffer for at least 5 min.
4. The membrane was wet in methanol for 5 min.
5. Membrane should uniformly change from opaque to semi-transparent.
6. The membrane was carefully placed in Milli-Q water and soaked for 2 min with the help of forceps. The membrane was carefully placed in transfer buffer and let equilibrate for at least 5 min.
7. The cassette holder was opened and a foam (fiber) pad placed on one side of the cassette.
8. One sheet of filter paper was placed on top of the pad.
9. On top of the filter paper the gel was placed.
10. On top of the gel the membrane was placed.
11. A second sheet of filter paper on top of the stack was placed.
12. On top of the filter paper second foam pad was placed.
13. To ensure an even transfer, air bubbles were removed by carefully rolling a pipette or a stirring rod over the surface of each layer in the stack.
14. The cassette holder was closed.

### 3.2.13.4 Protein Transfer

1. The cassette holder was placed in the transfer tank so that the gel side of the cassette holder is facing the cathode (–) and the membrane side is facing the anode (+).

2. Adequate amount of buffer was added to the tank to cover the cassette holder. The anode lead and cathode lead were connected to their corresponding power outputs. The system was turned on for 1 to 2 h at 6 to 8 V/cm inter-electrode distance and the tank manufacturer's guidelines were followed (Mini Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad)

**3.2.13.5 Immunodetection**

After performing electro transfer of proteins to PVDF, the membrane was incubated with antibody specific to the protein of interest for detection of transfer.

**3.2.13.6 Reagents**

1. Primary antibody (specific for protein of interest).
2. Secondary antibody labeled with horseradish peroxidase.
3. DAB (3, 3′ diaminobenzidine).
4. 50 mM Tris base, pH 7.6.
5. 30% $H_2O_2$
6. Washing buffer: Phosphate buffered saline (PBS), 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl.
7. Blocking solution: 3% (w/v) gelatin in PBS, 0.05% Tween-20.
8. Methanol, 100%
9. Milli-Q water.
10. Substrate: DAB/$H_2O_2$: (A) 5 mg DAB dissolved in 10 ml of 50 mM of Tris base, pH 7.6; (B) 30 ml water + 20 µl $H_2O_2$ (30%).

**3.2.13.7 Procedure**

After electro blotting, membrane was carefully placed in a blocking buffer (3% gelatin in PBS) at 37 °C for 2 h to block the non-specific binding sites. The membrane was washed thrice with washing buffer (PBS) and transferred into anti-

AGP monoclonal antibody LM-2 antibody solution (1:500 dilution in 0.5% BSA in PBS) for 1 h at 37 °C. Again, the membrane was washed thoroughly with washing buffer (PBS) and incubated with goat anti-rabbit IgG-HRP conjugate (1:5000 dilution) for 1 h at 37 °C. The membrane was washed thrice with washing buffer and the blot was developed with developing solution, (A) 5 mg DAB dissolved in 10 ml of 50 mM of Tris base, pH 7.6 (B) 30 ml water + 20 µl of 30% $H_2O_2$. Developing solution was prepared by mixing equal volume of A and B, and immunoblot was developed in dark.

### 3.2.14 Immunogold localization of AGP protein

For Immunogold localization of AGP protein, three different samples were taken from stem. All three tissues were fixed in 1% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M (pH 7.2) sodium-cacodylate buffer. After rinsing in the same buffer, the tissues were fixed in 1% aqueous $OsO_4$ for 1h at room temperature. Samples were then rinsed in water and dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 95 and 100 each for 10 min). The dehydrated tissues were infiltrated and embedded in LR white resin, which was polymerized for 16 h at 60 °C. Thin sections were cut with an ultramicrotome (Reichert, OMU3, Austria).

Ultrathin sections were mounted on 200-300 mesh coated gold grid for immunogold labeling. The grids were placed on drops of blocking solution, 5% (w/v) low fat milk powder for 10 min, followed by washing in 10mM phosphate buffer saline pH 7.4, for 20 min. The primary antibody was diluted to 1:500 in PBS containing 1% (w/v) low fat milk powder and incubated for 2 h at room temperature. The sections were washed three times in PBS (15 min each) and incubated for 1h with secondary antibody, rat anti-rabbit IgG conjugated with 10 nm gold particles (Amersham Pharmacia Biotech), diluted 1:500 with 1% ( w/v) low fat milk powder in

PBS. The grids were finally washed three times in PBS for 15 min per rinse. All the samples were observed using a Zeiss EM 906 transmission electron microscope.

## 3.3 Results

### 3.3.1 Purification and quantification of AGP

Purification of AGP was carried out by using Yariv reagent that is specific for AGP, as described in Section 3.2.4. The quantification of AGP and its derivatives was done using gel diffusion assay containing Yariv reagent (fig-3.1). On comparing zone of diffusion, the concentration of AGP was found to be 4 mg/ml. It is clear from the result that Yariv reagent reacted with all AGP preparation and form halo zone (fig. 3.1, A, AGP 1, 2, 3, AGP–AmB 1, 2, 3), while no halo zone develop with non-AGP preparations (fig- 3.1, B, C and AmB).

### 3.3.2 SDS-PAGE Protein Profile

The AGP was isolated from groundnut seedling and analyzed on 10 % SDS- PAGE. Multiple bands were observed in coomassie blue stained gel of crude protein isolated from groundnut seedling (fig-3.2, A), alcian blue staining revealed few bands of glycoprotein (fig-3.2, B). Protein profile of purified AGP is shown in fig 3.3 (lane-B) whose molecular weight was found to be 46 kDa when compared with protein standard. AGP presence was further confirmed by yariv in gel staining (fig 3.3, lane-C) and by performing western blotting using monoclonal antibody LM-2 (fig 3.3, lane-D).

### 3.3.3 Immunolocalization of AGP

For localization of AGP in cell, samples were taken from shoot of groundnut plant and immunogold microscopy was employed. The polyclonal antibody raised in rabbit, against purified AGP was used as probe. The immunolocalization of AGP is shown in fig-3.4. The AGP was found to be attached with plasma membrane of cell.

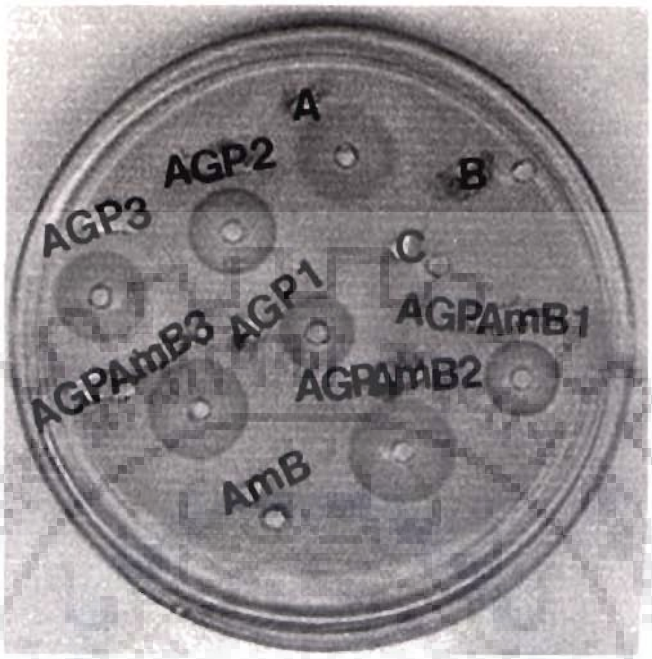



**Figure 3.1** Radial gel diffusion assay of AGP using Yariv reagent. Agarose gel (1%) containing 0.02% Yariv reagent, 0.15 M NaCl, 0.02% sodium azide was poured into Petri dish. After solidification, wells (4mm dia) were made and loaded with respective samples. AGP1, AGP2 and AGP3 are 15 µl, 30 µl (4mg/ml), and 30 µl (4mg/ml) after six-month storage of AGP, respectively. AGP-AmB1, AGP-AmB2 and AGP-AmB3, are 15 µl, 30 µl (4mg/ml) and 30 µl (4mg/ml) after six-month storage, of AGP-AmB conjugate, respectively. A-30 µl (4mg/ml) of Gum Arabic as positive control, AmB, B and C are, amphotericin B, buffer alone and 30 µl (4mg/ml) of larch arabinogalactan as negative controls, respectively.

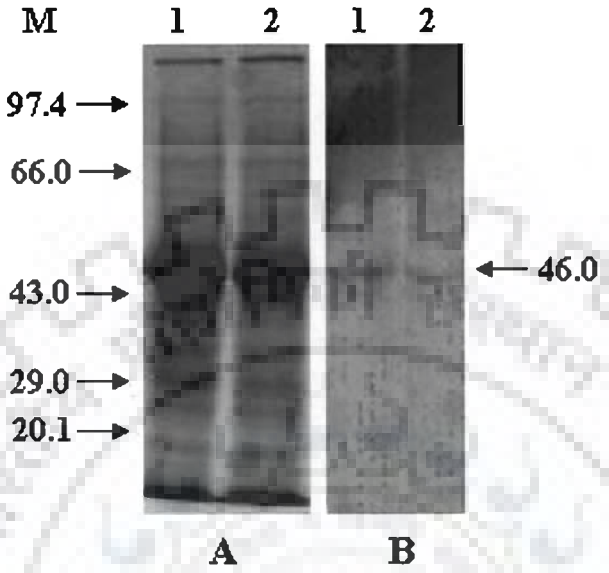



**Figure- 3.2** Coomassie blue (A) and alcian blue (B) stained SDS-PAGE (10 %) protein profile of AGP crude fraction. Lane 1 & 2 are same samples run in duplicate and stained with coomassie blue and alcian blue respectively. Molecular weights of the standard in kDa are shown on the left side.

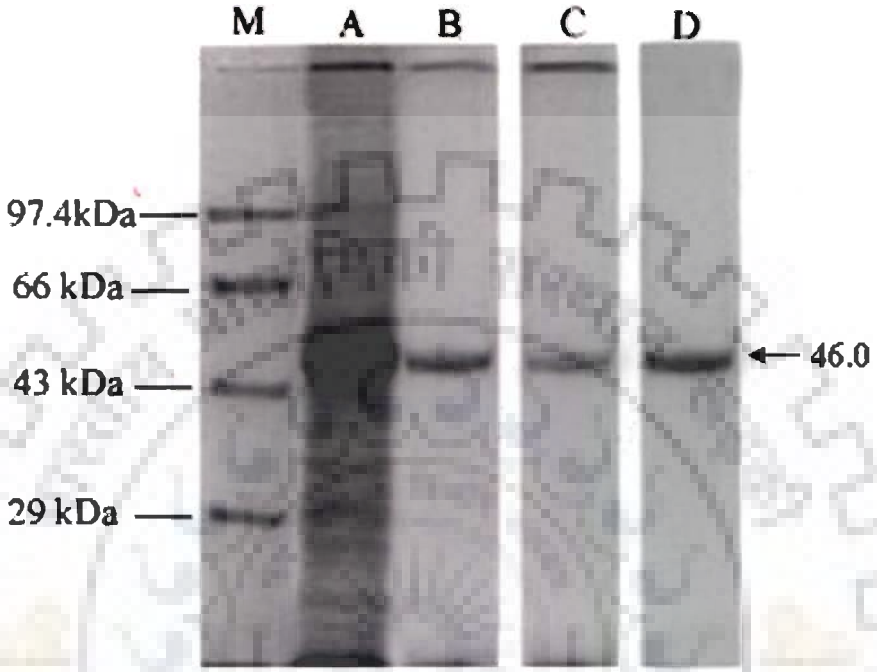



**Figure- 3.3** SDS-PAGE (10%) profile of crude and purified 46 kDa AGP fractions. A- coomassie blue stained gel of crude fraction, B- coomassie blue stained purified 46 kDa AGP, C- Yariv reagent stained purified 46 kDa AGP and D-western blot of purified 46 kDa AGP with anti-AGP monoclonal antibody LM-2. 30, 10, 10 & 10 µg of protein were loaded in lanes A, B, C & D respectively. Molecular weight standards are shown on left side.

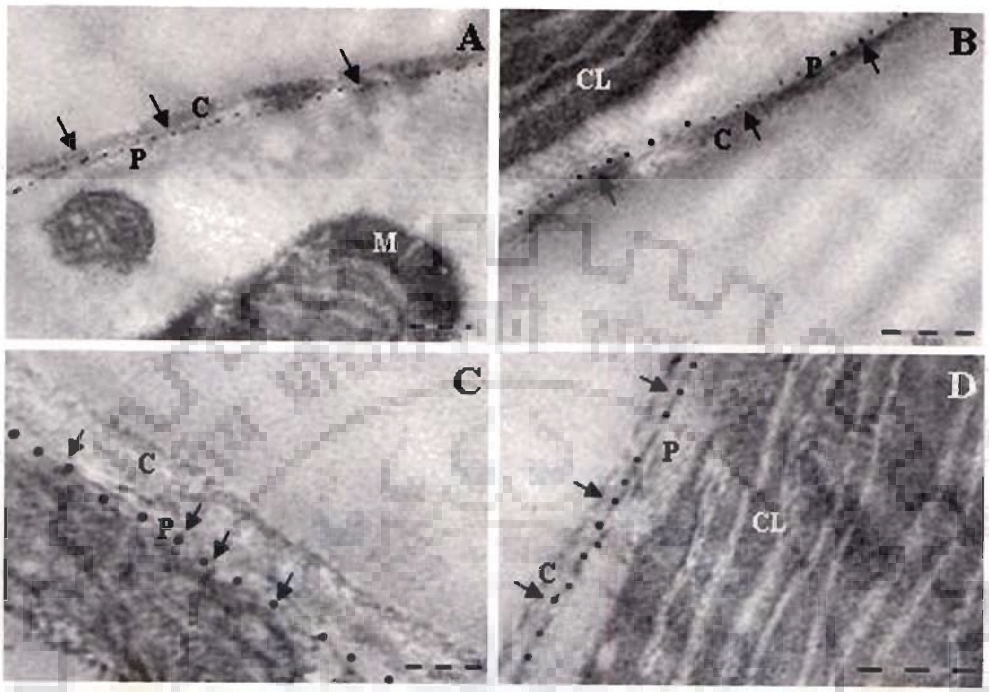



**Figure- 3.4** Immunogold localization electron micrograph of purified 46 kDa AGP in stem cell of groundnut seedling. Primary antibody used was anti-AGP polyclonal antibody raised against purified 46 kDa AGP. Gold particle label are shown with arrows. Various sub cellular organelles present are mentioned. C-cell wall, P-plasma membrane, M- mitochondria & CL-chloroplast. A-Bar represent 0.5 μm, B-Bar represent 0.5 μm, C-Bar represent 0.2 μm & D-Bar represent 0.5 μm.

## 3.4 Discussion

The study of AGPs in past have been relatively difficult task due to lack of precise procedure to get homogenous protein. But discovery of an important class of molecular probes for the investigation of AGPs by Yariv *et al.*, (1967) has been a major breakthrough in AGP research. The general chemical name for this class of intensely red-colored probes is 1,3,5-tri-(p-glycosyloxyphenylazo)-2,4,6-trihydroxy benzene. Although the common names "Yariv antigens" and "Yariv reagents" have been widely used for these probes but the name "Yariv phenylglycosides" is more appropriate.

The chemical synthesis of Yariv phenylglycosides was originally reported by Yariv *et al.*, (1962). Since then numerous Yariv phenylglycosides carrying different sugars in α- or β- anomeric configuration have been synthesized and tested for binding to AGPs (Yariv *et al.*, 1967; Jermyn and Yeow, 1975; Nothnagel and Lyon, 1986). The consensus of these and other studies is that two structural features of Yariv phenylglycosides are fundamental requirements for the interaction with AGPs. First, for D-absolute configuration sugars, the glycosidic linkage must be in β-anomeric configuration. Second, carbon atom two in the sugar must have a hydroxyl group in the D-gluco configuration. Thus, $(\beta\text{-D-Glc})_3$ and $(\beta\text{-D-Gal})_3$ Yariv phenylglycosides precipitate AGPs but the $(\alpha\text{-D-Gal})_3$ and $(\beta\text{-D-Man})_3$ Yariv phenylglycosides do not. The precise nature of interaction of Yariv reagent with AGPs is not well known and there is inconsistency in observation. Partial degradation of AGPs isolated from *Alocasia macrorrhizos* and rose implicated that the carbohydrate portion of the AGPs interacts with Yariv phenylglycosides (Komalavilas *et al.*, 1991). On the other hand, an AGP from the style of Gladiolus retained the ability to bind $(\beta\text{-D-Glc})_3$ yariv reagent after complete removal of arabinofuranosyl residues by enzymatic cleavage (Gleeson and Clarke, 1979), and a grape AGP treated with both

α-L-arabinofuranosidase and endo-(1-6)-β-D-galactanase still gave a positive reaction with $(\beta\text{-D-Glc})_3$ yariv reagent (Saulnier *et al.*, 1992). Likewise, radish AGP retained the ability to bind Yariv phenylglycoside after treatment with α-L-arabinofuranosidase, after carboxyl-reduction of glucuronosyl residues, or after Smith degradation of the peripheral regions of the carbohydrate chains (Tsumuraya *et al.*,1987). These latter results suggested that the polypeptide portion of AGPs was most important with regard to interaction with Yariv phenylglycosides. Thus, partial degradation has not led to a consistent conclusion as what part of the AGP interacts with Yariv phenylglycosides.

Due to the specificity of $(\beta\text{-D-Glc})_3$ and $(\beta\text{-D-Gal})_3$ Yariv phenylglycosides for AGPs, these are commonly used for detection, purification and functional study of AGPs from various plants (Willats and Knox, 1996; van Hengel and Roberts, 2002; Park *et al.*, 2003; Lee *et al.*, 2005; Qin and Zhao 2006; Coimbra *et al.*, 2007). In the present study the $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside was used for AGP purification. The proteins were extracted using the extraction conditions which facilitate AGP extraction and its reaction with Yariv reagent. The crude protein fraction had several proteins as revealed from coomassie blue stained gel, while the alcian blue stained gel showed relatively few glycoprotein in preparation. The alcian blue staining of the gel indicated the presence of glycoprotein in crude extract and the presence of AGP was detected by gel diffusion assay. The AGP from crude extract was purified by Yariv reagent precipitation and a major AGP of 46 kDa was obtained. Though the protein was purified using Yariv reagent, its AGP nature was also confirmed by gel staining with Yariv reagent and also by it recognition with a AGP specific monoclonal antibody LM-2, a kind gift from Dr J. P. Knox (University of Leeds, UK)

A macromolecule is considered as AGP if it has both carbohydrate and polypeptide components and the carbohydrate component is rich in galactosyl and arabinosyl residues (Fincher *et al.*, 1983; Nothnagel, 1997). Furthermore, the galactosyl residues in AGPs occur predominately in (1, 3)-β-D, (1, 6)-β-D and (1, 3, 6)-β-D-galactopyranosyl linkage. These linkage are characteristic of type-II arabinogalactans (Carpita and Gibeaunt, 1993) and distinguish AGP from other arabinogalactans found in plants and microorganisms (Daffe *et al.*, 1993) which has predominately (1, 4)-β-D-galactopyranosyl linkage which does not react with Yariv reagent. Although in the present work no detailed study regarding sugar analysis has been performed. Since it has reacted with $(\beta\text{-D-Glc})_3$ Yariv phenylglycoside and also with AGP specific monoclonal antibody LM-2, therefore, it is indeed an AGP.

The AGPs are highly polymorphic molecules and show variations in both protein and carbohydrate components among various AGPs. However, there is inconsistency in the nomenclature of AGPs. Depending upon the protein and sugar contents, AGPs could be named as proteoglycans or glycoproteins. Most of the AGPs typically consist of about 10% protein and about 90% carbohydrate, the latter being present in the form of large polysaccharide chains usually named as proteoglycans. On the other hand, it should be noted that some AGPs or AGP-like molecules with unusually high protein contents in the range of 30% (Norman *et al.*, 1990) or even 65% (Cheung *et al.*, 1995) have been reported, and these macromolecules are classified as glycoproteins (Pennell *et al.*, 1989; Pennell, 1992; Chasan, 1994). The AGP purified in the present study belongs to the later group, this is mainly because it has protein content in the range of 60% and sugar in the range of 40% as observed from protein and sugar estimation. This was also reflected in the relatively sharp or less diffused band in gel as observed by staining with coomassie blue and Yariv

reagent. On the other hand, relatively more diffused bands were obtained with proteoglycan due to high content of sugars.

AGPs are found to be present at different subcellular locations, in particular cell types and tissues such as on the outer surface of the plasma membrane (Knox *et al.*, 1989; Serpe and Nothnagel, 1996; Yang and Showalter, 2007), bound to the cell wall (Serpe and Nothnagel, 1999; Gao and Showalter, 2000) or as soluble molecules in periplasmic space and plant secretions (Pereira-Netto *et al.*, 2007). Each of these is partially distinct and has partially overlapping structures. The localization at different location has functional significance. Therefore, in order to assign the possible role of AGPs, it is important to know their localization inside cell. Immunolocalization study using various anti-AGP specific monoclonal antibodies has been the method of choice to determine the sub cellular distribution. Though the distinction between bound and soluble AGPs could be made easily using light microscopy but it could not differentiate between the cell wall and membrane bound AGPs. Immunogold labeling study using electron microscopy has been used for reliable distinction between labeling in the cell wall and plasma membrane. Localization of cell wall bound and plasma membrane bound AGPs from various plants and tissues have been studied using immunogold labeling. Immunogold labeling study using electron microscopy of the 46 kDa AGP protein isolated in present work showed that it is a plasma membrane bound AGP. Membrane bound AGPs have been reported from several plants and their function has been studied. Membrane bound AGPs are known to play important role in cell signaling, pollen tube germination (Jauh and Lord, 1996) and somatic embyogenesis inductions (Letarte *et al.*, 2006; Pereira-Netto *et al.*, 2007). The possible function of the 46 kDa AGP obtained in present work need to be explored. Its role in growth and somatic embryogenesis has been described in later chapter.

It is concluded that a 46 kDa AGP from groundnut seedling was isolated and purified using $(\beta\text{-D-Glc})_3$ yariv reagent. Based on its protein and sugar content it belongs to glycoprotein class rather than proteoglycan and found to be localized on plasma membrane.

# PART- B

## EXPRESSION OF 46 kDa AGP UNDER VARIOUS ABIOTIC STRESSES

### 4.1 Introduction

Environmental factors such as biotic (various pathogens) and abiotic stresses (drought, salt, temperature, soil pH) are found to have great influence on crop production and yield. Therefore, to cope up with the ever increasing demand for food, the development of disease resistant and abiotic stress tolerant varieties of crops have been the thrust area globally. Among abiotic stresses, salinity is one of the major factors affecting crop growth and productivity. Soil salinity is found to have adverse affects on survival, biomass production and productivity of most of the crop plants (Francois and Maas, 1994; Khanna-Chopra *et al.*, 1998; Grover *et al.*, 1998; Khush *et al.*, 1998; Sairam and Tyagi, 2004; Parida and Das, 2005; Brini *et al.*, 2007).

In order to develop salt tolerant plants by genetic manipulation either through molecular marker assisted selection, generation of transgenic plants by introducing novel gene or by altering the expression level of existing genes, it is essential to understand the molecular basis of salt stress responses of the plants. Study of salt stress response has been one of the thrust areas of research globally and attempts are being made to understand the molecular and biochemical basis of stress responses of various important crops and plants (Holmstrom *et al.*, 2000; Mahalingam *et al.*, 2003; Branco *et al.*, 2004; Cao *et al.*, 2006; Brini *et al.*, 2007). Ion transporter proteins, antioxidant enzymes (Gossette *et al.*, 1994; Benavides *et al.*, 2000; Demiral and Turkan, 2005) and accumulation of compatible osmolytes like proline (Hong *et al.*, 2000; Jain *et al.*, 2001; Khedr *et al.*, 2003), glycinebetaine (Paleg *et al.*, 1984;

Xinghong and Congming, 2005; Demiral and Turkan, 2006), mannitol, sorbitol etc (Cui *et al.*, 2004) are reported to play important role in salt tolerance of several plants. Recent advances in molecular tools and technologies have been helpful to identify a number of salt stress responsive proteins and their respective genes (Sreenivasulu *et al.*, 2004; 2007; Munns, 2005). Transgenic plants with enhanced salt tolerance have been produced using candidate genes in number of plants including *Arabidopsis* (Karakas *et al.*, 1997; Hmida-Sayari *et al.*, 2005; Cho *et al.*, 2006; Chen *et al.*, 2007 a; b). There are several excellent reviews discussing the problems and advancements made in the salt stress tolerance (Sairam and Tyagi, 2004; Parida and Das, 2005; Ashraf and Harris, 2004). Presently much emphasis has been given to identify suitable molecular markers or biomarkers from various important crop plants and use them for development of salt tolerant crops.

Arabinogalactan proteins (AGPs) play important role in various cellular processes. There are several reports regarding the role of AGPs in cell growth, differentiation and development from various important crops (Du *et al.*, 1996a; Wu *et al.*, 2001; van Hengel and Roberts, 2002; Shibaya and Sugawara, 2007). These are described in more detail in review literature section. The expression of AGPs have been found to be affected by biotic and abiotic stress, wounding, pathogen invasion, heat shock and phytohormones (Li and Showalter, 1996; Gilson *et al.*, 2001; Park *et al.*, 2003; Sun *et al.*, 2004) and the correlation of AGPs with salt stress have been reported (Zhu *et al.*, 1993; Lamport *et al.*, 2006). In two different studies performed on tobacco salt adapted and un-adapted cell lines, great variations in AGPs were observed and there possible correlations with salt stress have been discussed (Zhu *et al.*, 1993; Lamport *et al.*, 2006). Therefore, attempts were made to study the

expression of AGP in various abiotic stress conditions, using various cultivars of groundnut and to explore its possible role in salt stress.

## 4.2 Materials and methods

### 4.2.1 Materials

All chemicals and solvents used in the present study were obtained from M/s Sigma-Aldrich (USA), Pierce Chemicals (USA), Merck India, BDH (India) and SRL (India). Specific chemicals were purchased from their respective sources such as Freund's adjuvant from Sigma-Aldrich (USA), and goat anti rabbit IgG-HRP conjugate from Santa Cruz Biotech. Inc. (USA). Groundnut (*A. hypogaea* cv. Kaushal) seeds were obtained from the National Seed Centre, Roorkee, Uttarakhand, India and TG-64, TMV-7, ICGS-37, Jawan, KRG-1, TG-1, Tirupati-1, Kadri-4 and Tirupati-4 cultivars of groundnut were obtained from National Research Centre for Groundnut, Junagarh, Gujrat, India.

### 4.2.2 Pretreatment of seeds and growth conditions

Seeds were pretreated and grown as described previously (section 3.2.2)

### 4.2.3 Study the 46 kDa AGP expressions in various groundnut cultivars under salt and other abiotic stresses

#### 4.2.3.1 Abiotic stress treatment

To study the effect of increasing salt concentration and other abiotic stress on the AGP expression, the seedlings of Kaushal cultivar of groundnut were grown hydroponically on nutrient medium as described in section 3.2.2, supplemented with either different NaCl concentration (0, 50, 100, 150 and 200 mM) or different chemicals viz. PEG (20%), mannitol (300 mM), NaCl (150 mM), KCl (150 mM) and ABA (20 μM) respectively. The effect of salt stress on AGP expression in different cultivars of groundnut (viz. TG-64, TMV-7, ICGS-37, Jawan, KRG-1, Kaushal, TG-1, Tirupati-1, Kadri-4 and Tirupati-4), was studied by growing them under

150 mM NaCl stress conditions as described in section 3.2.2. The expression was studied using anti-AGP polyclonal antibody generated against purified AGP as described previously (3.2.11).

#### 4.2.3.2 Extraction and estimation of protein

The total protein extraction of seven days old control and seedlings grown under different abiotic stress conditions were performed as per method described (Aarati *et al.*, 2003), with slight modifications. Seedlings were washed with double distilled water, cut into small pieces and powdered in liquid nitrogen using mortar pestle. The powder was homogenized in (1:1 w/v) extraction buffer (Tris–HCl buffer (50 mM, pH 8.0), 1 mM PMSF, 5 mM β-mercaptoethanol, 1 mM EDTA) at 4 °C and centrifuged at 7,000xg for 40 min at 4 °C. Supernatant was collected for further experiments. The protein in the preparation was estimated as described earlier and either used directly or stored in aliquots at -80 °C so that it can be used later for expression studies.

#### 4.2.3.3 AGP expression using western blotting

Expression of the purified AGP under various experimental conditions mentioned above was performed by western blotting as per method described in previous section (3.2.13). However, the primary antibody used for the expression studies was Anti-AGP polyclonal antibodies raised against purified AGP in place of Anti-AGP monoclonal antibody LM-2.

## 4.3 Results

### 4.3.1 Expression of 46 kDa AGP under different NaCl concentrations

The expression of AGP was studied by using anti-AGP polyclonal antibody raised against 46 kDa AGP. Gradual decrease was observed in the expression level of 46 kDa protein with increasing NaCl concentration. The expression level was highest in control seedlings and lowest in 200 mM NaCl treated seedlings as exhibited by

coomassie blue stained gel and western blot (fig. 4.1 A & B, lane 1-5). This was also revealed by quantitative densitometry analysis of gel as well as the blot (fig. 4.1 C & D). The level of expression of protein was observed as approximately 28%, 23%, 18%, 15% and 12% in 0, 50, 100, 150 and 200 mM NaCl stress, respectively.

### 4.3.2 Expression of 46 kDa AGP under various abiotic stresses

The expression of 46 kDa AGP was found to be affected by various other abiotic stresses like KCl, Mannitol, Polyethylene glycol (PEG) and also in presence of abscisic acid (ABA). There was repression of AGP expression under different abiotic stresses tried and the level of expression differ greatly among them. (fig. 4.2 A & B). In case of PEG stress, the expression level of 46 kDa AGP was minimum which showed that protein is maximum repressed by PEG stress (lane 6) as compared to control seedling which has the highest expression. The expression was almost similar in NaCl and KCl treated seedling as shown in fig. 4.2 B (lane 2 & 3). On the other hand there was a little decrease in mannitol treated seedling and almost no decrease in ABA treated samples compared to control. (fig. 4.2 B, lane 4 & 5). These results were also supported by quantitative densitometry analysis which showed that level of expression was approximately 22% in control, 21% in ABA stress seedlings, 19% in mannitol stress, 16-17% in NaCl and KCl stress and 5.9% in PEG stress (fig. 4.2 C & D, lane1-6).

### 4.3.3 Expression of 46 kDa AGP in different cultivars of groundnut under salt stress

The effect of NaCl stress on the expression level of 46 kDa AGP was also studied in ten other cultivars of groundnut. Seedlings of all cultivars were grown under control and salt-stress (150 mM NaCl) condition for seven days and protein extract isolated from each sample was used for western blot analysis. Different level of expression patterns were observed in all cultivars (fig. 4.3). Differences were

observed in level of expression among various cultivars, both in control and salt treated seedling. However, the expression was repressed in salt treated seedlings of each cultivars compared to control. The expression level of 46 kDa AGP in control and salt-treated seedlings in five cultivars viz. T-64, TMV-7, ICGS-37, JAWAN and KRG-1 was shown in fig. 4.3 A & B. The change in level of expression was observed maximum in control and salt-treated seedlings of ICGS-37 (lane 5 & 6) and minimum in T-64 (lane 1 & 2), which was approximately 12% and 3% respectively as shown by quantitative densitometry analysis (fig. 4.3C). It was also observed that the level of protein expression in control seedlings of all cultivars was also different. It was maximum in ICGS-37 (lane 5) and minimum in JAWAN, (lane 7) approximately 17% and 10% respectively as exhibited by quantitative densitometry analysis. Almost similar observations were achieved in the expression of AGP in control and salt stress seedlings of other five cultivars viz. Kaushal, TG-1, Tirupati-1, Kadri-4 and Tirupati-4 (fig. 4.4 A & B). Expression level of 46 kDa AGP repressed maximum in salt stressed groundnut seedling of Kaushal and TG-1 cultivar (fig. 4.4, lane 2 & 4) and minimum in Kadri-4 cultivar (fig. 4.4, lane 7 & 8). The difference in the level of expression of AGP was also observed in the control seedlings of all cultivars (fig. 4.4 B, lane 1, 3, 5, 7 & 9). Kaushal exhibited the highest level of expression and Kadri-4 exhibited lowest level (lane 1 & 7). Almost similar levels of expression were seen in Kaushal and TG-1 (lane 1 & 3) as well as in Tirupati-1 and Tirupati-4 (lane 5 & 9) as evident from densitometry analysis (fig. 4.4 C).

### 4.3.4 Expression of 46 kDa AGP in root and shoot tissue

The expression of 46 kDa AGP was also studied in root and shoot of control and salt treated seedlings using anti-AGP polyclonal antibody. The effect of NaCl on the expression level of 46 kDa AGP in root and shoot of seedlings is given in fig. 4.5.

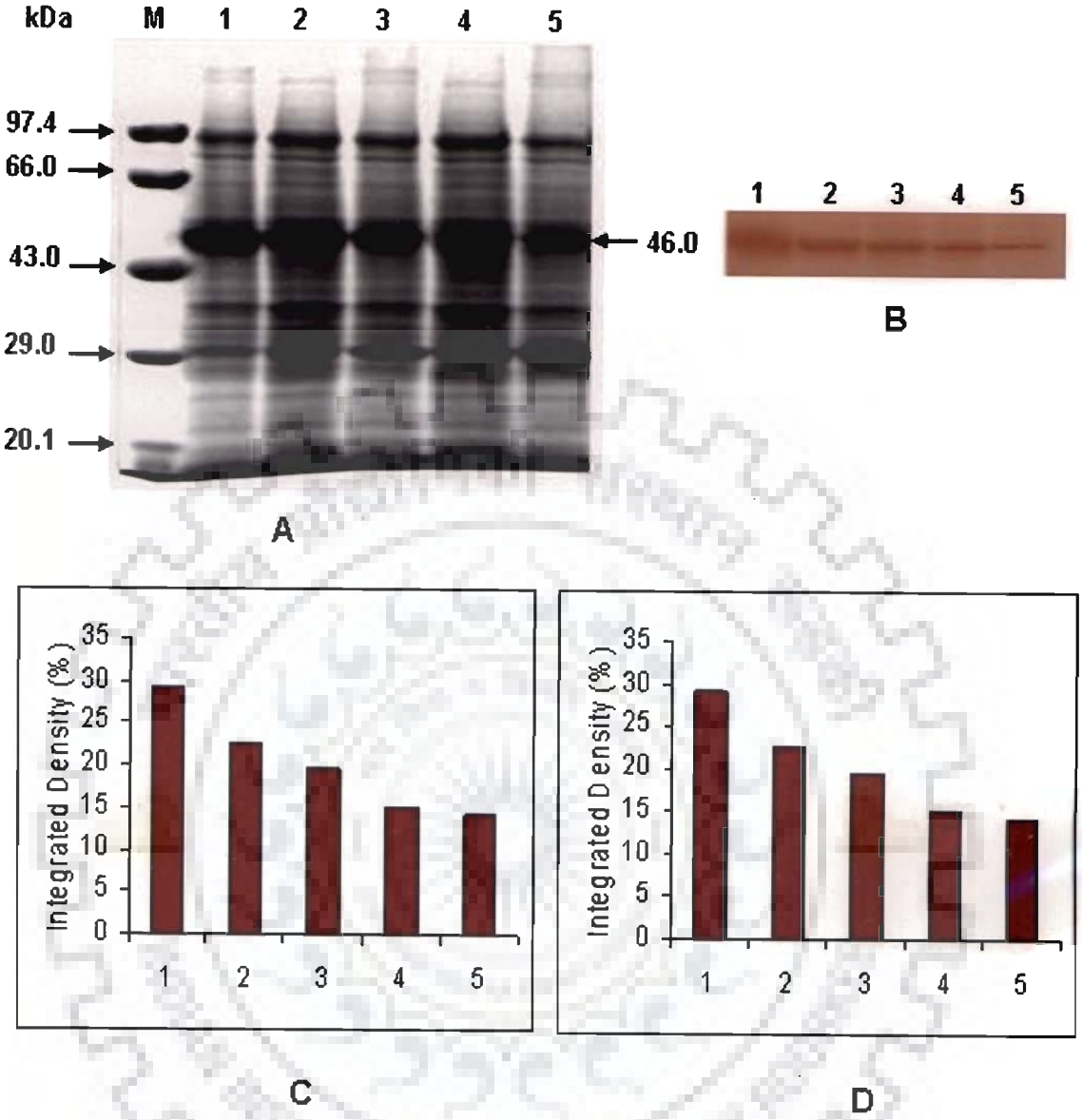



**Figure- 4.1** Expression of 46 kDa AGP in groundnut seedlings grown under different NaCl concentrations (0-200 mM). (**A**) Coomassie blue stained SDS-PAGE (10%) gel (**B**) Western blot using polyclonal antibody against 46 kDa AGP. Lane 1: Control; lane 2: 50 mM; lane 3: 100 mM; lane 4: 150 mM; lane 5: 200 mM NaCl grown seedlings.(**C**) and (**D**) are the densitometry analysis of 46 kDa AGP band from the (**A**) & (**B**), respectively. Molecular markers are shown at left hand side.

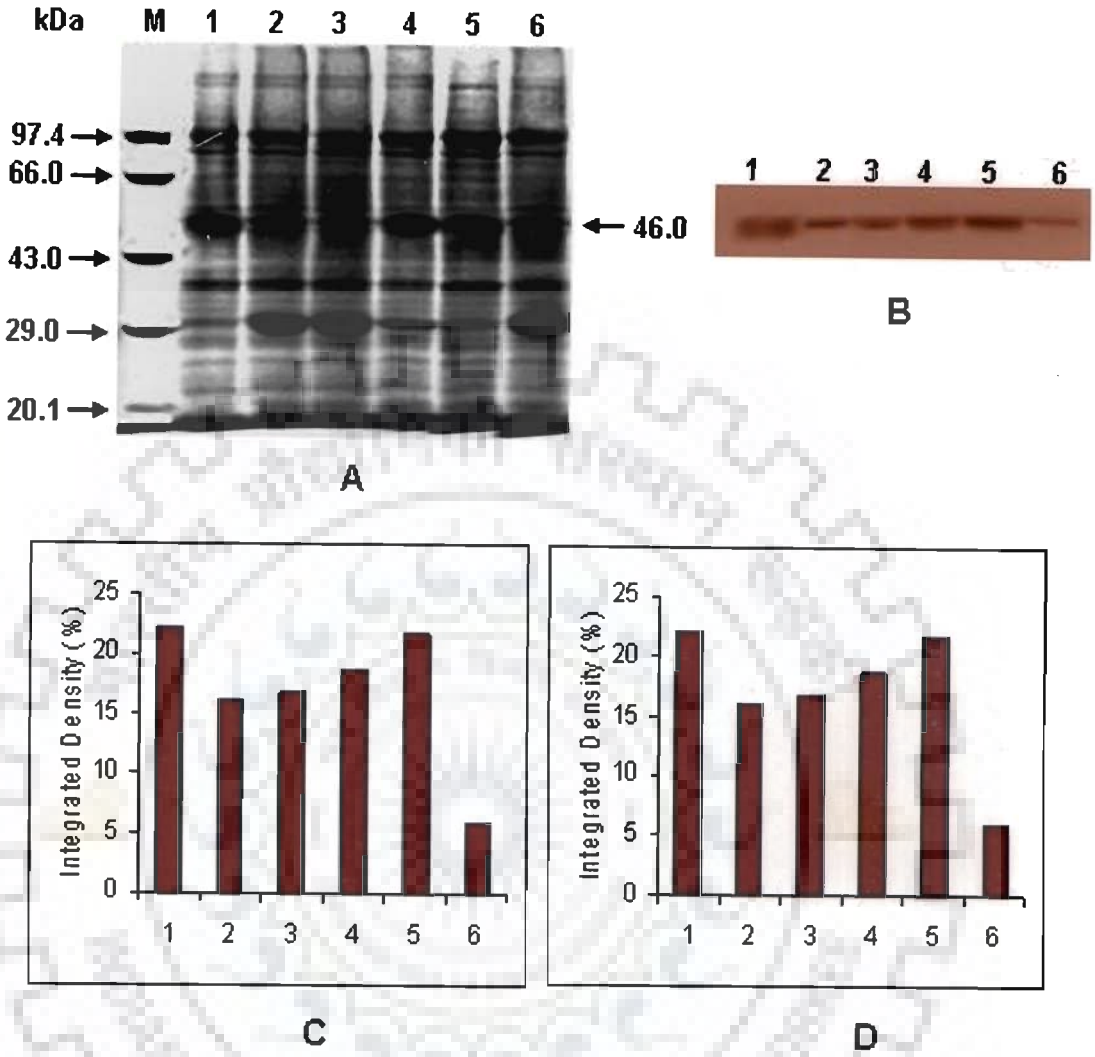



**Figure- 4.2** Expression of 46 kDa AGP in groundnut seedlings grown under various abiotic stresses and ABA (**A**) Coomassie blue stained SDS-PAGE protein profile of seedlings grown under different abiotic treatments (**B**) Western blot showing expression of the 46 kDa AGP under different abiotic treatments. Lane 1: Control; Lane 2: 150 mM NaCl; Lane 3: 150 mM KCl; Lane 4: 300 mM Mannitol; Lane 5: 20 µM ABA; Lane 6: 20 % PEG; (**C**) and (**D**) are the densitometry analysis of 46 kDa AGP band from the (**A**) & (**B**), respectively. Molecular markers are shown on left side.

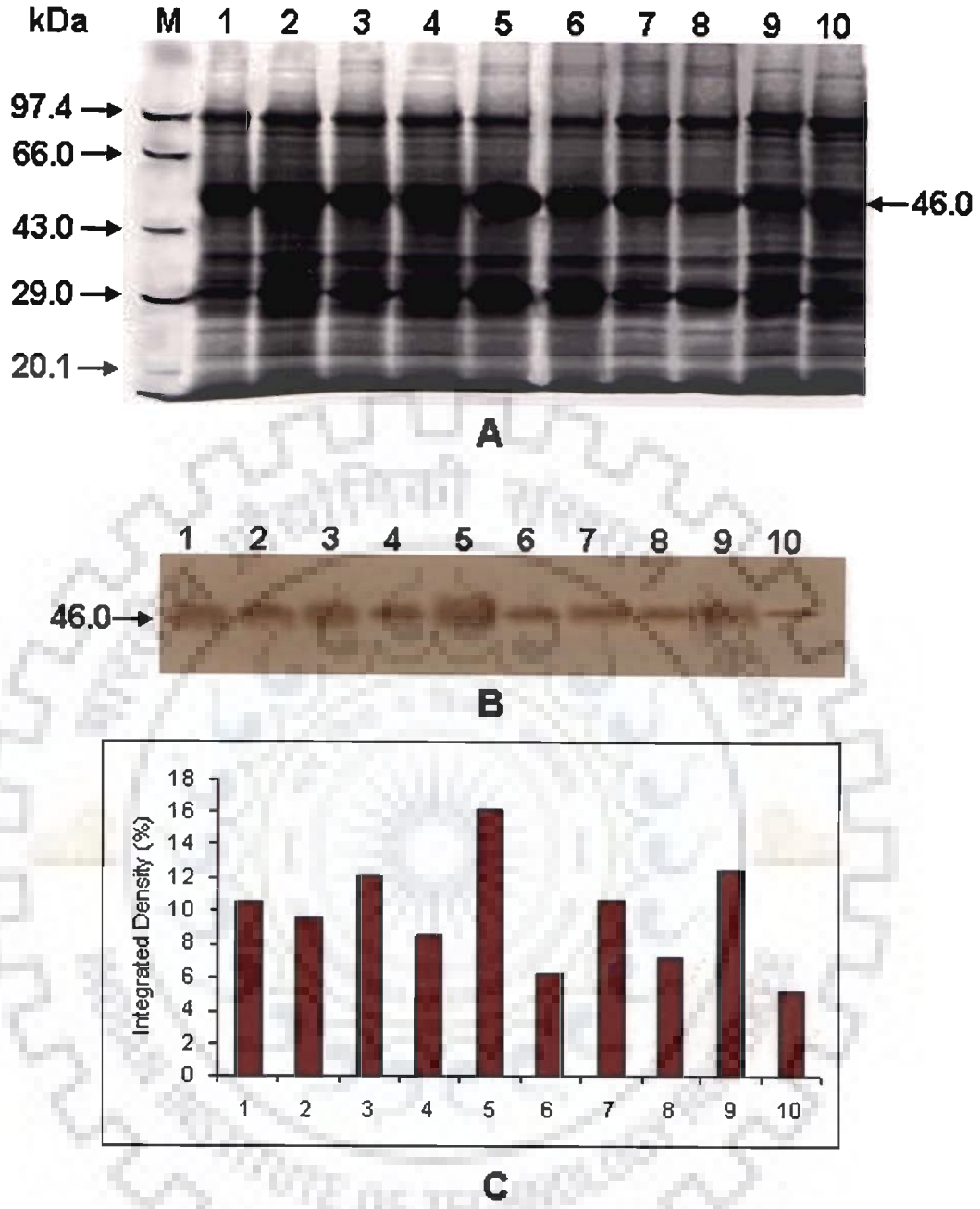



**Figure- 4.3** Expression of 46 kDa AGP in various cultivars of groundnut under NaCl stress. (A) Coomassie blue stained SDS-PAGE protein profile of various cultivars; (B) Western blot showing expression pattern of 46 kDa AGP protein in different cultivars of groundnut. Lane 1: T-64 control; lane 2: T-64 150 mM; lane 3: TMV-7 control; lane 4: TMV-7 150 mM; lane 5: ICGS-37 control; lane 6: ICGS-37 150 mM; lane 7: JAWAN control; lane 8: JAWAN 150 mM; lane 9: KRG-1 control; lane 10: KRG-1 150 mM; (C) The densitometry analysis of 46 kDa AGP protein band of the blot. Molecular markers are shown at left side

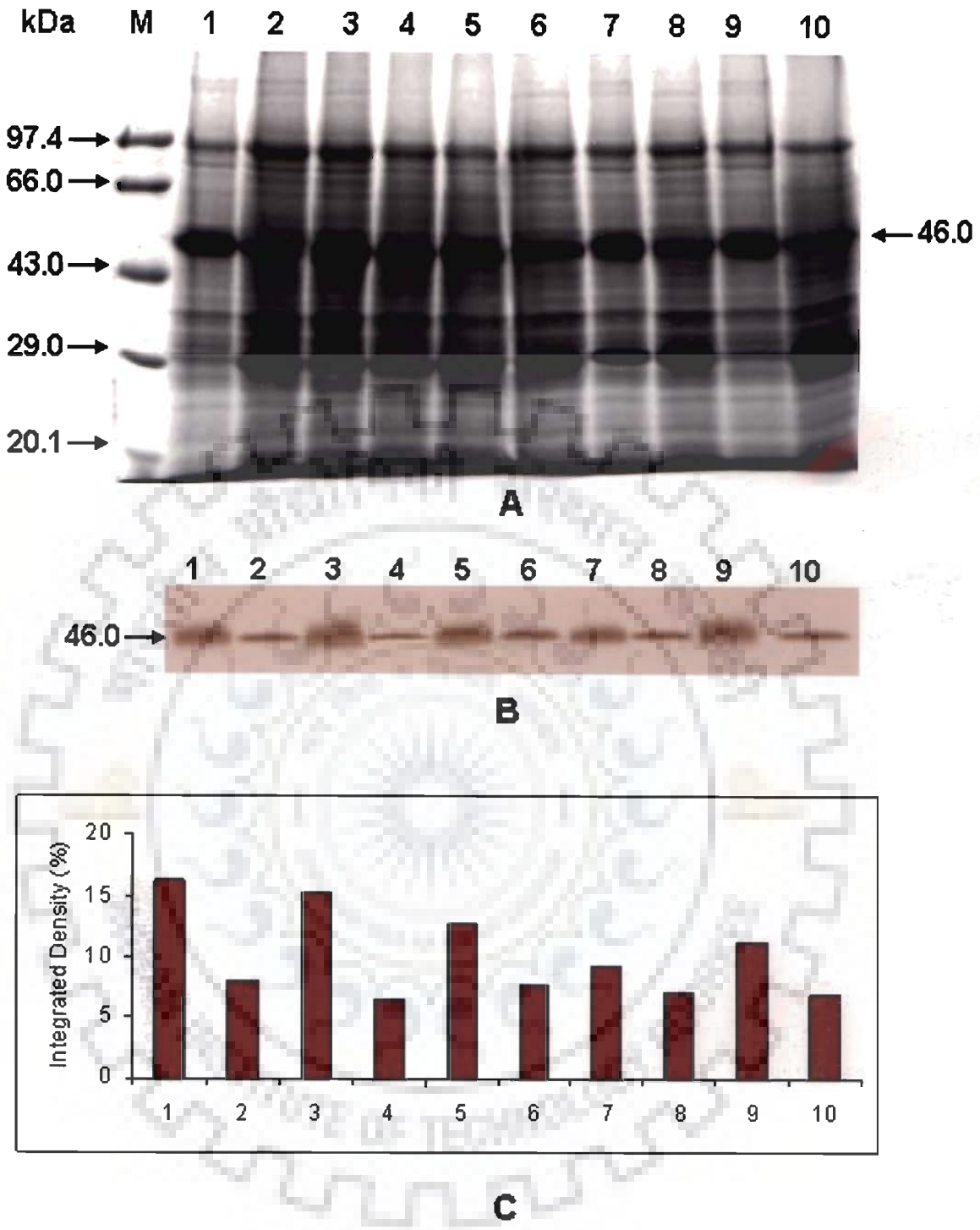



**Figure- 4.4** Expression profile of 46 kDa AGP in various cultivars of groundnut under NaCl stress (A) Coomassie blue stained SDS-PAGE protein profile of various cultivars, (B) Western blot showing expression pattern of 46 kDa AGP protein in different cultivars of groundnut. Lane 1: Kaushal control; lane 2: Kaushal 150 mM; lane 3: TG-1 control; lane 4: TG-1 150 mM; lane 5: Tirupati-1 control; lane 6: Tirupati-1 150 mM; lane 7: Kadri-4 control; lane 8: Kadri-4 150 mM; lane 9: Tirupati-4 control; lane 10: Tirupati-4 150 mM; (C) The densitometry analysis of 46 kDa AGP protein band of the blot.

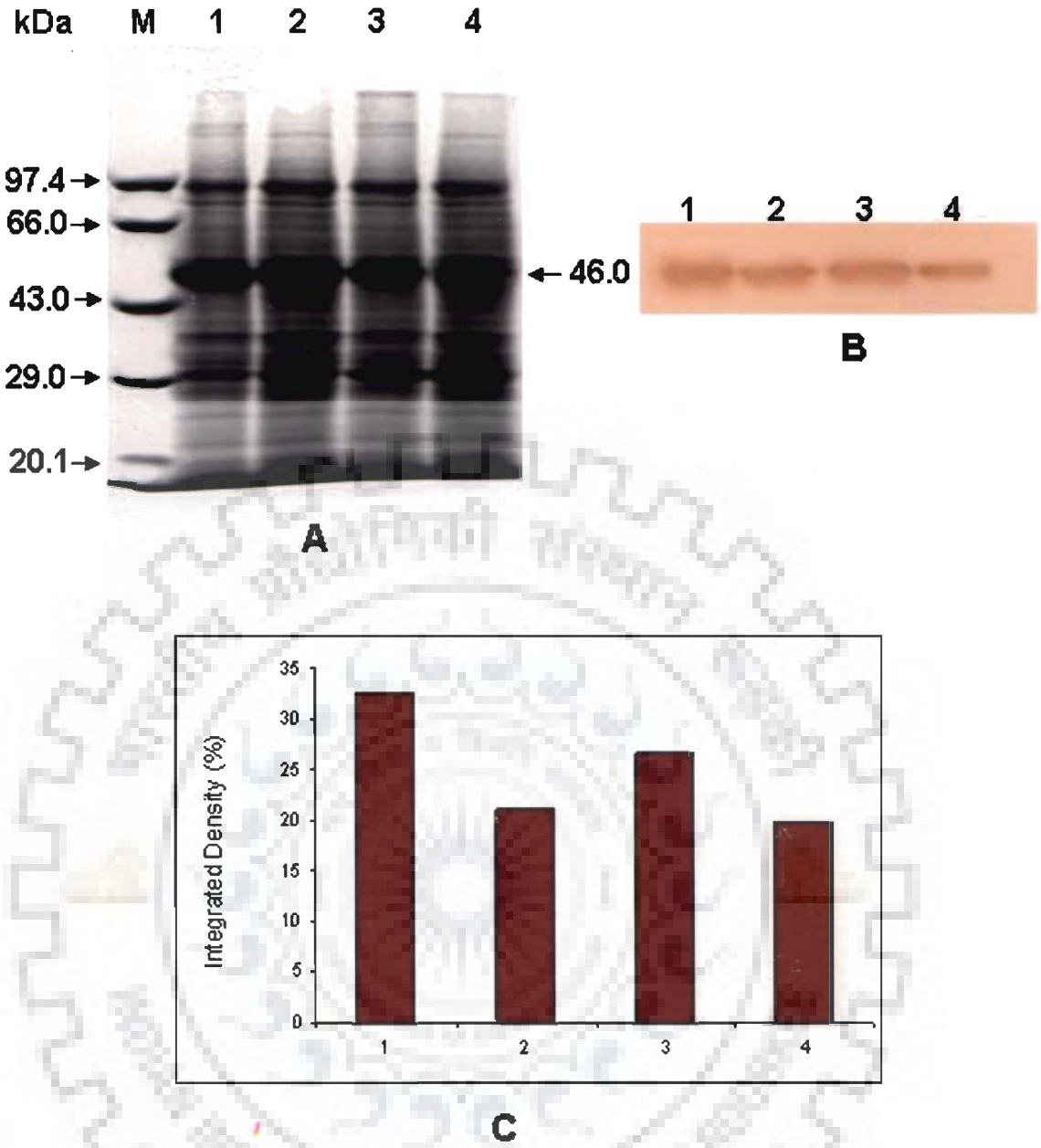



**Figure- 4.5** Tissue specific expression of salt repressed 46 kDa AGP in root and shoot of groundnut seedling. (A) Coomassie blue stained SDS-PAGE protein profile (B) Western blot using polyclonal antibody against 46 kDa AGP protein Lane 1: root control; lane 2: root 150 mM NaCl; lane 3: shoot control; lane 4: shoot 150 mM NaCl. (C) The densitometry analysis 46 kDa AGP protein band from the blot Molecular markers are shown at left hand side.

The level of expression of AGP has been repressed more in root than in shoot as shown in fig. 4.5 A & B. The expression of protein also showed differences in root and shoot of control seedlings. The level of expression in root was more than it was observed in shoot, approximately 32% and 27%, respectively (fig. 4.5 C, lane 1, 3).

## 4.4 Discussion

Salt stress found to have adverse effect on growth and development of plant and cell. This is associated with various molecular and biochemical alterations which take place under stress conditions. Alteration in protein biosynthesis is one of the major changes. Great variations were observed in control and salt treated samples protein profiles. Much emphasis has been given for identification of stress induced proteins and study of their possible role in salt tolerance. It was mainly due to the fact that these proteins are induced or formed under stress so they may have possible role in salt tolerance. In contrast there are very few studies on salt suppressed protein and their role in abiotic stress tolerance. One of such proteins whose expression reduced under salt stress is AGP. AGP's role in cell expansion and differentiation is well established. Zhu *et al.*, 1993 have reported the difference in the expression of tobacco salt adapted, slow growing and un-adapted, fast growing cell line. They reported the loss of AGP from plasma membrane of salt adapted cell compared to un-adapted and discussed its possible role in salt adaptation. The present study supports the earlier observations, though not performed on cell line but on seedling. The repression of 46 kDa AGP in salt stressed seedlings indicated the negative effect of NaCl stress on its expression. Different cultivars responded differently to NaCl stress on the basis of expression level of 46 kDa AGP. In order to see whether there is any link in level of

AGP expression and stress tolerance, the expression of AGP in control and salt treated seedling of drought tolerant and sensitive cultivars were analyzed carefully. It could be revealed from the expression profile of AGP of control and salt treated seedling of different cultivars in general and the drought tolerant cultivar ICGS-37 in particular (fig. 4.3 A & B, lane 5 & 6 ) that AGP expression is associated with stress tolerance. The significant low level of expression of AGP under salt and other abiotic stresses compared to control among different cultivars indicates that change in AGP expression is one of the major salt stress response and this may be one of the various possible mechanism of salt tolerance. This was further revealed from the observations that the AGP expression was more reduced in salt treated seedling of ICGS-37, a drought tolerant line compared to expression in its control seedling (fig. 4.3 A & B, lane 5 & 6). Perhaps this may be helpful in its drought adaptation. Since the salt and drought responses are very similar it might also be helpful in salt adaptation. But in present work no direct study was performed to validate this point.

The 46 kDa AGP was found to be present both in root and shoot tissues of seedling and its expression level in both the tissues under salt stress were compared to control. AGPs have been reported to be present in different types of organs and tissues and variations were found in their expression levels (Knox *et al.,* 1991; Motose *et al.,* 2004; Tang *et al.*, 2006). AGP was reported to function as an epidermal wall-loosening factor in auxin-mediated coleoptile growth in maize (Schopfer, 1990), it is possible that reduced level of AGP in root and shoot of seedling may be helpful for its salt adaptation. However, there are evidences to show that expression level of AGP is associated with salt adaptation. Cultured glycophyte cells adapted to desiccation stress or NaCl stress exhibit a reduced rate of cell enlargement relative to un-adapted cells even though osmotic adjustment has resulted in higher turgor than

pre-stress levels (Bressan *et al.*, 1982; Binzel *et al.*, 1985). This reduced cell-expansion rate has been hypothesized to be a result of decreased cell-wall extensibility or an altered wall loosening mechanism (Bressan *et al.*, 1990). Alterations were found in some physical and biochemical parameters of cell walls of the adapted cells like reduction in wall tensile strength, decrease in the mass of cellulose-extension network and organization of the peptic substances (Iraki *et al.*, 1989a-c). However it could not be proven that any of these changes participate directly in reducing the ability of the cell wall to extend, and the exact cause of the slow expansion in osmotically adapted cells.

However, based on the fact that AGPs play a role in plasma membrane-cell wall binding (Roberts, 1989) and function as an epidermal wall-loosening factor in auxin-mediated coleoptile growth in maize (Schopfer, 1990), its possible role in reducing the ability of the cell wall to extend which causes slow expansion in osmotically adapted cells has been suggested (Zhu *et al.*, 1993). They observed that salt adapted tobacco cells has much reduced level of membrane bound AGP compared to un-adapted cells. It was further observed that the reduction in the level of Yariv reagent reactive AGPs associated with the plasma membrane of slow growing salt adapted cells had substantial influence on the biophysical interactions between the plasma membrane and cell wall. It was suggested that AGPs may function as lubricating agents between cellulose microfibrils in the cell wall (Schopfer, 1990). Thus plasma-membrane AGPs play a lubricating role in the cell wall-plasma membrane slippage and hence these alterations in AGPs during stress adaptation could reduce cell growth. Recently, Lamport *et al.*, (2006) have studied the expression and fate of AGP in tobacco salt adapted and un-adapted cell line and they have observed the increase in the periplasmic AGP but decrease in the membrane bound

AGP in salt adapted cell line compared to un-adapted which have high level of expression of both form under salt stress. They discussed the possible role of membrane AGP in salt adaptation. Alternatively, AGPs might function in cell expansion in other ways. It has long been known that AGPs bind to Yariv reagents, synthetic molecules containing phenyl-β-glycosides (Yariv *et al.*, 1967; Clarke *et al.*, 1978). Although the mechanism of this binding is not well understood, it points to a possible function of cell-surface AGPs. Structures similar to phenyl-β-glycosides might be present in cell-wall polysaccharides and phenolics, so AGPs might be able to bind to these cell-wall components. Rohringer *et al.*, (1989) reported that AGPs isolated from leek seeds were able to bind to components of the cell wall of wheat cells, possibly β-linked bound sugars. It has been found that ferulic-acid glycoconjugates have structure similar to phenyl-β-glycosides of Yariv reagents, except that the sugars are ester-linked to the phenolics. Ferulic acid is ester-linked to the hemicellulosic polysaccharide arabinoxylan in the cell wall of monocotyledons, and to the pectic polysaccharide arabinogalactan in dicotyledons (Fry 1979; 1983; 1986). The bound ferulic acids are able to undergo oxidation to form diferulic acids. Diferulic acids cross-link cell-wall polysaccharides, thereby probably decreasing cell-wall extensibility. It is possible that cell-surface AGPs bind to linked ferulic acids in the cell wall to prevent formation of diferulic acids, thereby regulating wall extensibility. Loss of Yariv reagent reactive AGPs from the plasma membrane of salt adapted cells could lead to decreased wall extensibility of these cells.

Thus it is concluded that AGP play important role in salt adaptation of cells possibly by reducing the ability of the cell wall to extend which results in slow expansion under salt or other osmotic stress conditions and allow the plant to survive under limited water and nutrient.

# PART-C

## ROLE OF 46 kDa AGP IN SOMATIC EMBRYOGENESIS

### 5.1 Introduction

Being leguminous plant, groundnut plays an important role in biological nitrogen fixation thus increasing and maintaining soil fertility. Because of inappropriate production practices, susceptibility of cultivars to various abiotic stresses and largely because of damage of crop by several pathogens, there is a large gap between the potential yield and farm yield. The major production constraint is groundnut rosette disease, which is caused by a complex of two viruses, *groundnut rosette assistor luteovirus* (GRAV) and *groundnut rosette umbravirus* (GRV). Groundnut is susceptible to late leaf spot (LLS), caused by *Phaeoisasariopsis personata* and to rust disease, caused by *Puccinia arachidis* (Pande *et al.*, 2001). Another important productivity constraint is *Cercospora personata* which causes tikka disease of groundnut. So, there is an urgent need to improve groundnut cultivar grown in India or elsewhere. With the development of different methods for genetic transformation, it has been possible to introduce genes with characters beneficial for the plant. Recombinant DNA technology has greatly improved the quality of harvests by inserting genes that produce resistance to disease caused by various pathogens. Gene transformation methods allow introduction of new genes which are not normally accessible by conventional means.

Recently, development of genetic transformation technology and efficient systems of controlling morphogenesis from cells and tissues has been shown to have great advantage in crop improvement. The difficulty in regenerating many groundnut

plants from elite or mature phase selections is one of the most serious problems for applying gene transfer technologies to these plants. There is need for the development of an efficient regeneration system in groundnut. Somatic embryogenesis provides an ideal experimental process for investigation of plant differentiation as well as the large-scale production of plants. Regeneration of plants through somatic embryogenesis has been a preferred method for genetic transformation in plants because somatic embryogenesis leads to the formation of bipolar structures, possessing both shoot and root meristem. Presence of well developed root and shoot primordial in somatic embryos has also enabled them to germinate easily to produce entire plantlets without the additional step of rooting (Laux and Jugens, 1997). Somatic embryogenic systems can exhibit some distinct advantages when used for transformation. High number of regenerates can be achieved originating from few or single cell which increase the likelihood of achieving transformed plants. Many reports of somatic embryogenesis in groundnut have been developed using various explants like leaflet (Baker and Wetzstein, 1992; Venkatachalam *et al.*, 1999), mature zygotic embryo derived leaflet (Chengalrayan *et al.*, 2001), cotyledons (Baker and Wetzstein, 1995; Durham and Parrott, 1992), hypocotyls (Venkatachalam *et al.*, 1997), immature embryo (Peggy O-A, 1989) and epicotyl (Little *et al.*, 2000). In all these studies, number of somatic embryos obtained were low and were dependent on various combinations and concentrations of 2,4-D, NAA, kinetin, and BAP. Another important factor to be considered in all the aforesaid studies is time span taken to achieve somatic embryogenesis is eight or more weeks (Baker and Wetzstein 1994; Pacheco *et al.*, 2007). Therefore, search for potential macromolecules that could enhance number of somatic embryos and reduce the time of somatic embryo induction is still on. AGPs isolated from various sources were reported to have somatic

embryogenesis inducing potential and being used exogenously for study of somatic embryogenesis in different plants (Letarte *et al.*, 2006; Pereira-Netto *et al.*, 2007). The reduction of somatic embryos generation time and the increase in the number of somatic embryos developed under the influence of such somatic embryogenesis inducing agents, could be a boon for plant regeneration and may help significantly in crop improvement. The aim of the present study was to evaluate the somatic embryogenesis inducing potential of 46 kDa AGP isolated from *A. hypogaea.*

## 5.2 Materials and Methods

### 5.2.1 Materials

All the culture media components, hormones and other supplements used in the study were purchased from Hi-Media, India. All other chemicals used were of analytical grade.

### 5.2.2 Preparation of hormone stocks

The various hormones like α-naphthalene acetic acid (NAA), Kinetin, 6-benzyl aminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) used for callus formation, shooting and somatic embryogenesis study and solvent used for their preparation are listed below. Hormones (5 mg) were dissolved in minimum amount of solvent (1-2 ml) and the volume was made up to 5 ml with autoclaved distilled water so that the working concentration of all hormones was achieved as 1mg/ml.

| Hormone | Solubility | Heat Stability | Storage Temperature |
|---|---|---|---|
| NAA | 0.1N NaOH | Autoclaved | 4°C |
| 2,4-D | 0.1NNaOH | Autoclaved | 4°C |
| Kinetin | 0.1N NaOH | Autoclaved | 4°C |
| BAP | 0.1N NaOH | Autoclaved | 4°C |

### 5.2.3 Preparation of growth media

To study the effect of AGP on callus formation, shoot induction and somatic embryogenesis of groundnut, as culture media, basal MS (Murashige and Skoog, 1962) media was used. In this media, 440 mg/L $CaCl_2$. $2H_2O$, 0.8% agar, 3% sucrose and growth regulators were added extra.

### 5.2.4 Sterilization of seeds

Approximately 25 groundnut seeds were sterilized at a time. For sterilization, seeds were first washed with 70% ethanol for 2 min and were further sterilized by 0.1% $HgCl_2$ (100 mg $HgCl_2$ in 100 ml autoclaved distilled water) for 7 min. Seeds were thoroughly washed with autoclaved distilled water, 4 times. These seeds were transferred to fresh flask containing 75 ml autoclaved distilled water for presoaking for 16 h, at 28°C, in dark.

### 5.2.5 Effect of AGP on shoot induction

The role of 46 kDa AGP on shoot induction was studied by using groundnut cotyledons as explants. The cotyledon explants were obtained from surface sterilized seeds presoaked for 16 h in water under aseptic conditions. MS media, pH 5.8, containing 3% (w/v) sucrose, 0.8 % (w/v) agar and fortified with 0.5 mg/L BAP and 0.5 mg/L kinetin was used for study. Cotyledons were cut from all sides and in each plate 4 cotyledons were implanted. The experiment was set into three groups as per following plan:

1. **Control group -** MS media + 0.5 mg/L BAP and 0.5 mg/L kinetin hormone
2. **AGP group** - MS media + 0.5 mg/L BAP and 0.5 mg/L kinetin hormone + AGP protein (10 µg/ml)
3. **Anti-AGP antibody group** - MS media + 0.5 mg/L BAP and 0.5 mg/L kinetin hormone + AGP (10 µg/ml) + anti-AGP polyclonal antibody (10 µl/ml)

All explants were incubated at 25 ± 2 °C under a 16 h photoperiod and 80% relative humidity. Light was provided by cool white fluorescent lamps with an intensity of 60 E/$m^2$s. The cultures were monitored regularly to examine the shoot induction.

### 5.2.6 Effect of AGP on somatic embryogenesis

To study the effect of AGP on somatic embryogenesis, appropriate concentrations of different hormones for somatic embryogenesis were first standardized. Similar to the shoot induction study, cotyledons were used as explants for somatic embryogenesis studies. The cotyledons were cut from all the sides and were placed by abaxial side in contact with MS media having varying concentration of 2,4-D (10, 20, 30, 40, 50 mg/L) and NAA (10, 20, 30, 40 mg/L). Explants were incubated at 25 ± 2 °C under a 16 h photoperiod (Baker *et al.*, 1994). Light was provided by cool white fluorescent lamps with an intensity of 60 E/$m^2$s.

To study the effect of AGP on somatic embryogenesis, all other conditions were same except for the AGP and anti-AGP polyclonal antibody addition. The whole of the experiment was conducted in three groups as per following plan:

1. **Control group** - MS media + 20 mg/L 2,4-D
2. **AGP group** - MS media + 20 mg/L 2,4-D + AGP (10 µg/ml)
3. **Anti-AGP antibody group**- MS media + 20 mg/L 2,4-D + AGP (10 µg/ml) + anti-AGP polyclonal antibody (10 µl/ml)

Explants were incubated at 25 ± 2 °C under a 16 h photoperiod. Light was provided by cool white fluorescent lamps with an intensity of 60 E/$m^2$s. The somatic embryo formation was monitored regularly for eight weeks.

## 5.3 Results

### 5.3.1 Callus and shoot formation

The results of callus and shoot induction are summarized in table-5.1 and fig.- 5.1 & 5.2. The groundnut cotyledons explants were grown over MS media under different conditions either in presence of hormone alone or in presence of AGP. It was observed that all explants whether grown in presence of hormone alone or in presence of AGP form callus after seven day of incubation. However no shoot induction was observed in explants grown in hormone alone and grown in presence of anti-AGP antibodies after seven days of incubation. On the other hand all the explants grown in presence of 10 µg/ml AGP were able to form callus in 7 day time span, also in this group 20% of calluses were showing shoot induction at $7^{th}$ day. Only 10% of explants grown in presence of 10 µl/ml of anti-AGP antibody were able to form callus at $7^{th}$ day and none of the explants showed shoot induction (fig. 5.1).

Observation at $16^{th}$ day showed that, in control group (hormone alone) 35% explants were showing shoot induction while in AGP group 80% explants were showing the shoot induction. In the anti-AGP antibody group there was no increase reported in callus percentage even on $16^{th}$ day, callus percentage remains the same 10%. In this group none of the explants was able to produce shoot (fig.5.2).

**Table- 5.1** Effect of 46 kDa AGP on shoot induction from the cotyledon explants of groundnut.

| Test Condition | No. of Explants | 7th day* | | 16th day* | |
|---|---|---|---|---|---|
| | | Callus Induction | Shoot Induction | Callus Induction | Shoot Induction |
| 1.Control group (20 mg/L 2,4-D) | 20 | 20 | ---- | 20 | 07 |
| 2. AGP group (0.5 mg/L BAP and mg/L kinetin + 10 µg/ml AGP) | 20 | 20 | 04 | 20 | 16 |
| 3. anti-AGP antibody group (0.5 mg/L BAP and 0.5 mg/L kinetin + 10 µg/ml AGP + 10 µl/ml anti-AGP polyclonal antibody) | 20 | 02 | ---- | 02 | ---- |

*The experiment was performed in triplicate. The data shown here are of representative experiment.

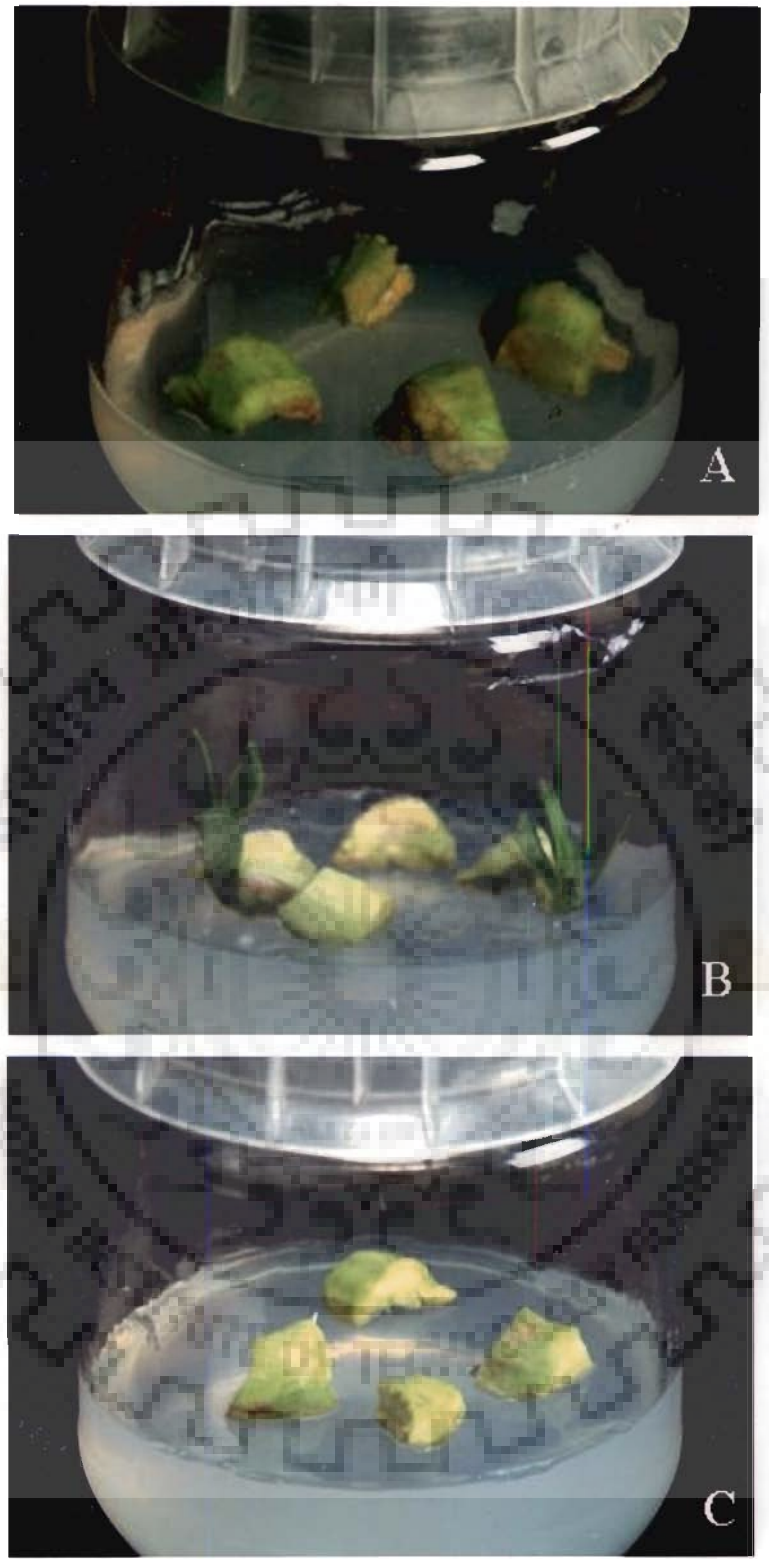



**Figure-5.1** Effect of 46 kDa AGP on callus and shoot induction in cotyledon explants at 7$^{th}$ day of culture.

A- control explants (0.5 mg/L BAP and 0.5 mg/L kinetin)

B- explants grown on 0.5 mg/L BAP and 0.5 mg/L kinetin + 10 µg/ml AGP protein

C- explants grown on 0.5 mg/L BAP and 0.5 mg/L kinetin + 10 µg/ml AGP protein + 10 µl/ml anti-AGP polyclonal antibody

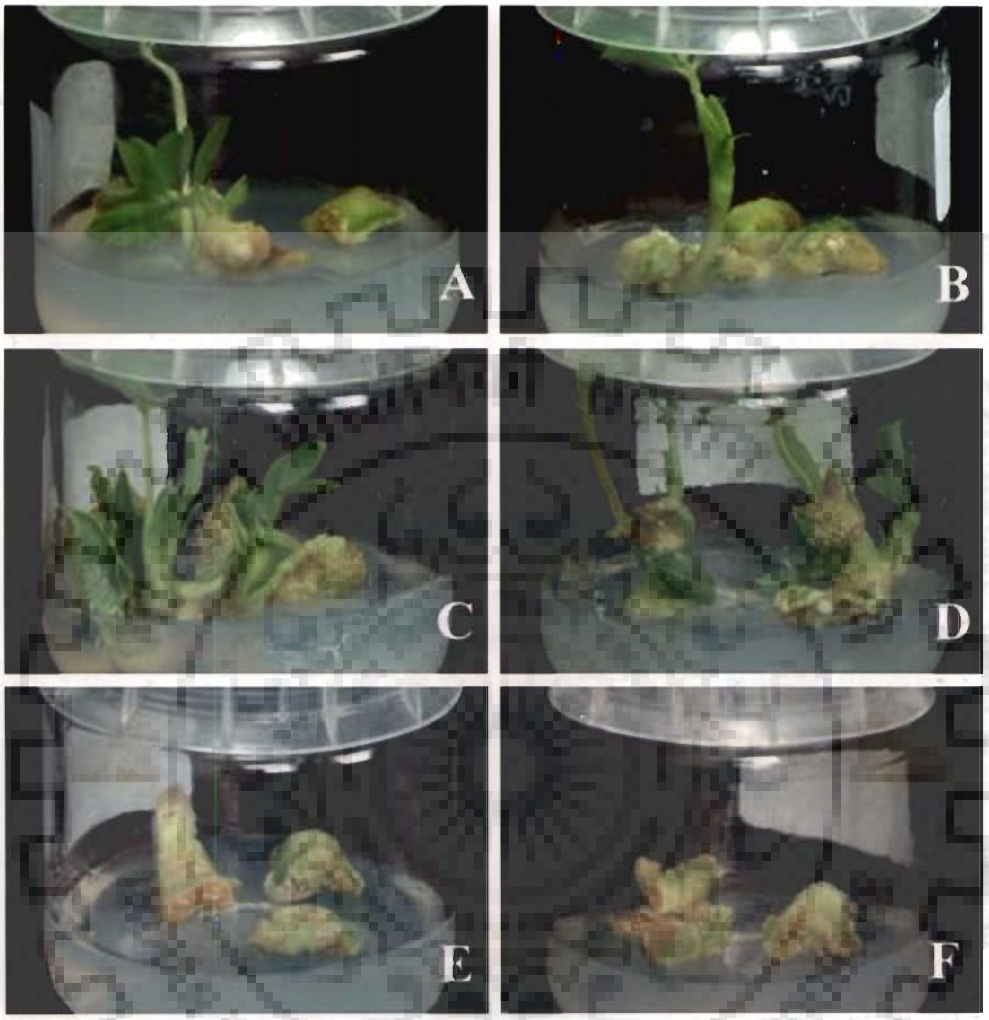



**Figure - 5.2** Effect of 46 kDa AGP on callus and shoot induction in cotyledon explants at 16$^{th}$ day of culture.

A, B - control explants (0.5 mg/L BAP and 0.5 mg/L kinetin)

C, D - explants grown on 0.5 mg/L BAP and 0.5 mg/L kinetin + 10 µg/ml AGP protein

E, F - explants grown on 0.5 mg/L BAP and 0.5 mg/L kinetin + 10 µg/ml AGP protein + 10 µl/ml anti-AGP polyclonal antibody

### 5.3.2 Effect of AGP on somatic embryogenesis induction

Hormones play an important role in growth and embryo development. Therefore, in order to study the effect of AGP on somatic embryogenesis induction, appropriate concentration of suitable hormone was first standardized and that concentration was used to study effect of 46 kDa AGP on somatic embryogenesis. The results of hormone standardization are shown in (table- 5.2 & 5.3). 2,4-D was found to be better hormone compared to NAA. The best concentration of 2,4-D was found to be 20 mg/L where percentage primary embryogenesis (81) and number of embryos per embryogenic explant (8.1) were maximum. Higher concentrations of 2,4-D have shown poor percentage primary embryogensis. The minimum value for primary embryogenesis (27) and number of embryos per embryogenic explant (4.6) were obtained at highest 2,4-D concentration used i.e 50 mg/L. Many explants on 40 and 50 mg/L 2,4-D were failed to form embryo. Another common feature observed was browning of explants which was increasing as going towards higher 2,4-D concentrations. Highest browning was exhibited by explants at 50 mg/L 2,4-D.

On the other hand, MS media containing different concentrations of NAA though showed induction of somatic embryos at all NAA levels (table-5.3) but the percentage of primary embryogenesis and number of embryos per embryogenic explant were low as compared to 2,4-D. The percentage of primary embryogenesis and number of embryos per embryogenic explants increases with increasing concentration of NAA up to 30 mg/L, but at 40 mg/L all these parameters have declined dramatically. The maximum percentage of primary embryogenesis (31) and maximum number of embryos per embryogenic explant (4.2) were obtained at 30 mg/L NAA level. The least value for percentage primary embryogenesis (17), and number of embryos per embryogenic explant (2.1) were achieved for 10 mg/L NAA

level. Browning of any explant was not observed at any of the NAA concentrations used.

Since, 2,4-D was found to be better hormone and 20 mg/L concentration was found to be appropriate concentration, effect of AGP on somatic embryogenesis induction and antagonistic effect of anti-AGP polyclonal antibodies were performed using this hormone concentration. Significant differences were observed in the results obtained from control group (hormone alone), AGP group and anti-AGP antibody group (table-5.4; fig. 5.3 and 5.4). The results indicated that AGP has somatic embryogenesis promoting effect. Use of 10 µg/ml AGP along with the 2,4-D in culture media increases the percentage primary embryogenesis to 97% in six weeks culture time, in comparison to 81% observed in control group after eight week culture time. The AGP also showed increase in the number of embryos per embryogenic explant to 12.1 in six week time in comparison to 8.1 embryos of control obtained in eight week culture time. On the other hand no somatic embryo could be seen in anti-AGP antibody group. In anti-AGP groups all the explants have turned brown in second week.

One very striking feature observed in this study is reduction of time span taken to achieve somatic embryogenesis. Using AGP along with 2,4-D hormone, somatic embryogenesis was observed in 6 weeks in comparison to 8 weeks of control (20 mg/L 2,4-D alone). In case of control group embryogenesis was observed only after 8 week culture time, at 6 week culture time, control explants were not showing any somatic embryogenesis.

**Table-5.2** Effect of 2,4-D on somatic embryogenesis for cotyledon explants after 8 week of culture time.

| Concentration of hormone (mg/L) | Percentage Primary embryogenesis | No. of embryos per embryogenic explant* |
|---|---|---|
| 10 | 74 | 6.9 ± 0.7 |
| 20 | 81 | 8.1 ± 0.3 |
| 30 | 60 | 5.6 ± 0.6 |
| 40 | 47 | 5.2 ± 0.6 |
| 50 | 27 | 4.6 ± 0.5 |

*The experiment was performed in triplicates. The data shown here are average value ± SE.

**Table-5.3** Effect of NAA on somatic embryogenesis for cotyledon explants after 8 week of culture time.

| Concentration of hormone (mg/L) | Percentage Primary embryogenesis | No. of embryos per embryogenic explant* |
|---|---|---|
| 10 | 17 | 2.1 ± 0.3 |
| 20 | 19 | 2.9 ± 0.3 |
| 30 | 31 | 4.2 ± 0.3 |
| 40 | 13 | 1.9 ± 0.2 |

*The experiment was performed in triplicates. The data shown here are average value ± SE.

**Table-5.4** Effect of 46kDa AGP on somatic embryogenesis for cotyledon explants.

| Group | Percentage Primary embryogenesis | No. of embryos per embryogenic explant* |
|---|---|---|
| 1.Control group (20mg/L 2,4-D) | 81# | 8.1 ± 0.3 |
| 2. AGP group (20 mg/L 2,4-D + 10µg/ml AGP) | 97## | 12.1 ± 0.4 |
| 3. anti-AGP antibody group (20 mg/L 2,4-D + 10µg/ml AGP + 10 µl/ml anti AGP polyclonal antibody) | 0.0 | 0.0 |

*The experiment was performed in triplicates. The data shown here are average value ± SE. # 8 week old culture, ## 6 week old culture

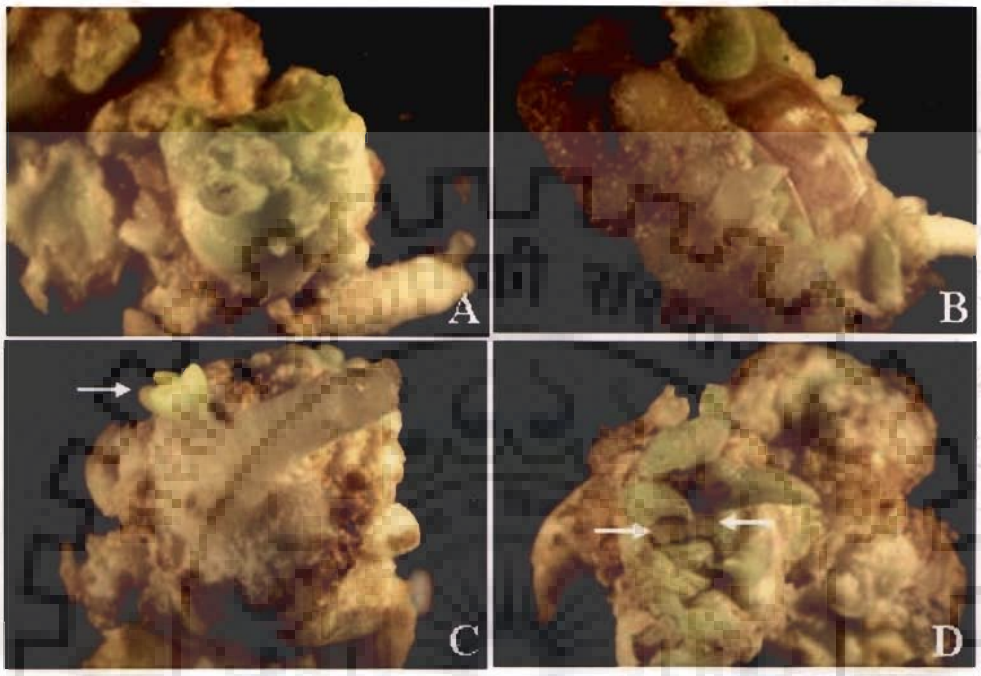



**Figure-5.3** Somatic embryogenesis in groundnut cotyledon explants in control culture in presence of 2,4-D (20mg/L) only.
A & B- 6 week explants (callus formation, no somatic embryogenesis seen)
C & D- 8 week explants (callus, some somatic embryos formed seen)

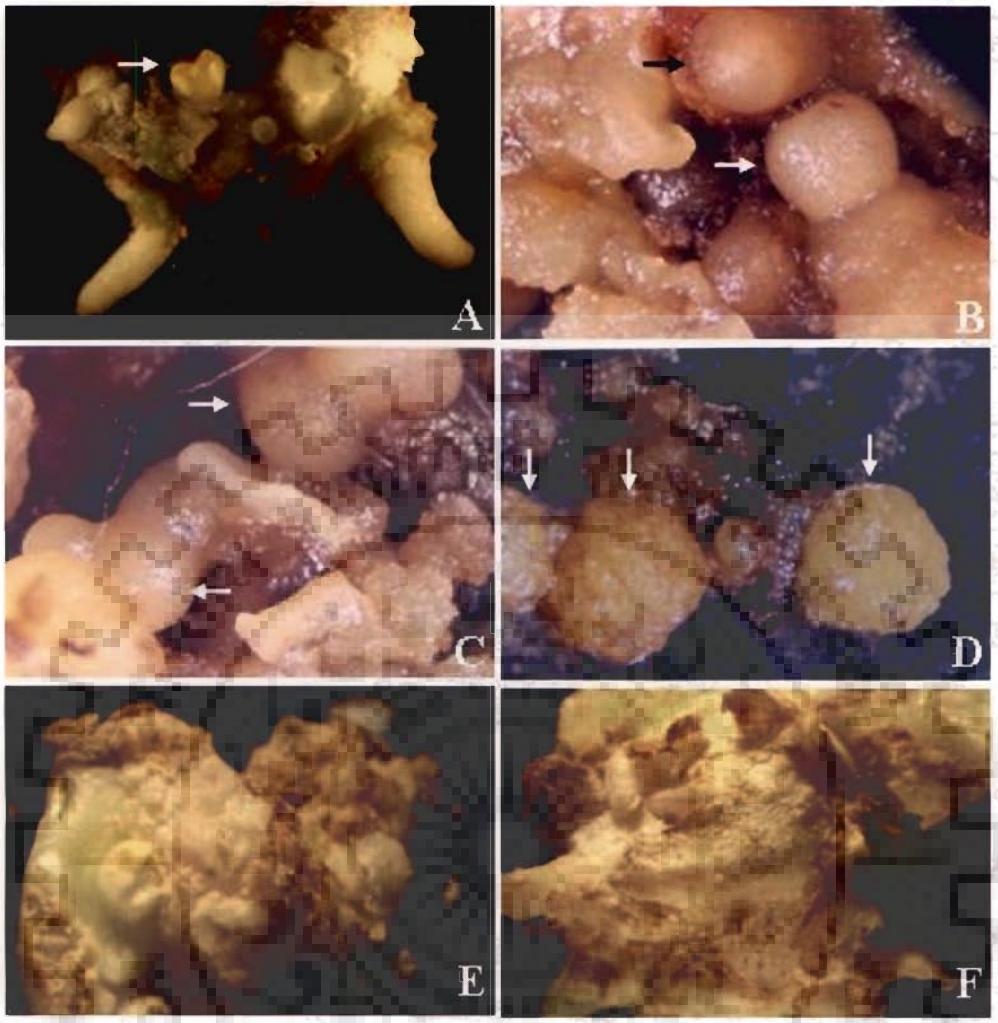



**Figure-5.4** Effect of 46 kDa AGP on somatic embryogenesis after 6 week culture time. A, B, C & D- explants grown in MS media in presence of 2,4-D (20 mg/L) along with 46 kDa AGP (10 µg/ml); E & F- explants grown in MS media in presence of 2,4-D (20 mg/L), AGP (10 µg/ml) and anti-AGP polyclonal antibody (10 µl/ml). Somatic embryos are shown by arrows (A, B, C & D). Somatic embryos could not be seen in anti-AGP antibody containing culture (E & F), instead callus turn brown in two weeks

## 5.4 Discussion

Hormones plays critical role in cell growth, development and somatic embryogenesis. Variations in action have been observed depending on both the hormone type and its concentration. Therefore, it was logical to first standardize the suitable hormone and its appropriate concentration for somatic embryogenesis induction study. It was clear from the result that 2,4-D is better hormone for somatic embryogenesis induction in groundnut and the 20 mg/L is the most suitable concentration. Suitability of 2,4-D as hormone for somatic embryogenesis study have also been reported from earlier studies. Venkatachalam *et al.*, (1999) observed that be among 2,4-D and NAA, 2,4-D was more effective and highest percentage of somatic embryogenesis was achieved at 20 mg/L of 2,4-D using leaflet explants. Hazara *et al.*, (1989) have seen that 2,4-D is essential for achieving direct somatic embryogenesis in cultivar SB-11 using immature zygotic embryo axis as explants. McKently *et al.*, (1991) have found that NAA was less effective than 2,4-D and picloram with embryo axis as explants. Only 12% of groundnut explants produced embryos with NAA than to 53% of explants induced on 2,4D. In the present study out of all the concentration tested, 20 mg/L 2,4-D have produced highest 81% primary embryogenesis. Venkatachalam *et al.*, (1997) also have reported that 20 mg/L 2,4-D was giving highest frequency of somatic embryos (62.7%) using hypocotyls as explants.

Therefore, the effect of AGP on somatic embryogenesis was studied using these parameters. Addition of AGP in medium along with 2,4-D, causes significant increase in somatic embryos formation compared to 2,4-D alone. On the other hand, the addition of anti-AGP antibody in medium totally suppress the somatic embryo induction, even though 2,4-D was present. This looks surprising but seems logical. It seems that anti-AGP antibody inactivated both the exogenously added AGP and also

endogenously expressed AGP if any. It has been reported that AGP is involved in auxin dependent cell growth and developmental process (Schopfer, 1990). It is likely that 2,4-D might have induced somatic embryogenesis by AGP expression or in association with auxin mediated cell signaling. The inactivation of endogenous AGP by anti-AGP antibody may be the reason for total inhibition of the somatic embryogenesis even in presence of 2,4-D. This need to be further investigated. Besides, increasing the number of embryos, the AGP addition also reduced the time of somatic embryos induction compared to 2,4-D alone. Thus both early induction, increase in number of embryo formation and total suppression of somatic embryogenesis in presence of anti-AGP antibody, confirmed the somatic embryogenesis promoting potential of 46 kDa AGP isolated from groundnut. Exogenously supplied AGPs, at the micromolar and nanomolar range, have been shown to change differentiation patterns in plant cells (Kreuger and van-Holst, 1993). In the past few decades, most of the work targeted to elucidate the role of AGP in plant cell growth and development has been focused on the effect of AGPs. However the mechanism of action of AGPs in somatic embryogenesis is not fully elucidated. van Hengel *et al.*, (2001) have suggested that whole AGP molecule as well as polysaccharide derived from it, might display different signaling roles, which might stimulate somatic embryogenesis by contributing to the establishment of cell identity and mechanism of cell to cell signaling. It has also been reported that AGP function as epidermal wall loosening factor in auxin mediated coleoptile growth in maize (Schopfer, 1990). Since the 1980's, oligosaccharides have been implicated as signals able to regulate physiological and developmental patterns in plants (van Hengel *et al.*, 2001; Ryan and Farmer, 1991). Recognition events employing purified oligosaccharides indicates that these molecule interact with cellular membrane

receptor in a hormone like way to change pattern of gene expression (Ryan and Farmer, 1991) and specifically induced signaling events (Vargas-Rechia *et al.*, 1998; Kuchitsu *et al.*, 1997).

Similar results have been reported in *Picea abies* where AGP, isolated from seeds was found to induce formation of more developed somatic embryos in low embryogenic cell lines (Egertsdotter and von Arnold, 1995). Kruger and van Holst (1995) have reported that ZUM18 AGP has increased the percentage of embryogenic cells from 40% to 80% with in one week, when ZUM18 AGP is added in to carrot cell culture. Kruger and van Holst (1993) have observed that addition of carrot seed AGP to two year old non embryogenic carrot cell line has resulted in re-induction of embryogenic potential. AGPs have been reported for inducing somatic embryogenesis in wheat microspore culture (Letarte *et al.*, 2006). Thompson and Knox (1998) have observed that when early stage embryos were grown in presence of yariv reagent there were no shooting induced from early stage embryos. Lee *et al.*, (2005) have shown that AGP is playing role in extension of apical tip cells in *Physcomitrella patens* moss. They explain that knocking out the AGP1 gene has resulted in reduced cell length in protonemal filament. The explants grown on MS media containing anti AGP polyclonal antibody were not able to show any callusing as well as shooting, suggesting the binding of anti-AGP antibody to AGP which cease the growth and development of cell.

When anti-AGP antibody was added into the MS media, no somatic embryogenesis was observed. Chapman *et al.*, (2000) have reported reversible effect of yariv reagent on somatic embryogenesis. They have observed that yariv reagent (250 μM) has completely blocked somatic embryogenesis in *Cichorium* but effect can be reversed by transfer of cells in to normal media. Since anti-AGP antibody binds

specifically to AGP and no somatic embryogenesis was observed, it can be said that AGP is essential for induction of somatic embryogenesis. In anti-AGP antibody group negligible growth was observed, which is demonstrating the role of AGP in growth. These results are similar to *Arabidopsis* where AtAGP19 functions in various aspects of plant growth and development, including cell division and expansion, leaf development and reproduction (Yang *et al.*, 2007). Explants grown on anti-AGP antibody containing media have turned brown in second week of culture, which is supporting that AGP is important for growth. Chaves *et al.*, (2002) have reported that yariv reagent is inducing programmed cell death in tobacco BY-2 suspension cultured cells. In *Arabidopsis* culture, programmed cell death has been reported as consequence of yariv reagent and AGP binding (Gao and Showalter, 1999). Inhibitory role of yariv reagent in somatic embryogenesis has been proved in *Daucus carota* also (Thompson and Knox, 1998). Tang *et al.*, (2006) revealed that β-glucosyl yariv treatment disturbs both the normal process of embryogenesis and the basic structural pattern of the embryos in *B. napus*.

In the present study, role of 46 kDa AGP in shoot induction from cotyledon explants of groundnut is explored and efficient protocol has been proposed for induction of somatic embryos using cotyledon explants with higher primary somatic embryogenesis in culture time less than eight weeks. Attempts were made to establish the promoting effect of 46 kDa AGP on groundnut somatic embryogenesis. Thus, it is concluded that the 46 kDa AGP have somatic embryogenesis potential by increasing the number of embryos formed and by reducing the time of somatic embryogenesis induction compared to normal hormones. This is the first report of 46 kDa AGP isolation from groundnut seedling and providing evidence for its role in shoot induction and somatic embryo induction in groundnut.

# PART- D

# ANTIMICROBIAL POTENTIAL OF 46 kDa AGP

## 6.1 Introduction

All living organisms including humans and plants are constantly exposed to potential harmful pathogens through out their life. This result in various diseases which have great impact on human health (Brogden *et al.*, 2005; Ghosh *et al.*, 2006) and crop productivity (Umesha, 2006; Rajarajeswari and Muralidharan, 2006). The animals and plants have evolved defense mechanism to overcome these problems. More often, this first line of defense mechanism failed and various chemotherapeutic agents and antibiotics are to be used to combat the infections caused by pathogens. In past, a large number of chemical agents and antibiotics have been discovered or synthesized in order to treat and cure these infections. However, widespread and indiscriminate use of these conventional drugs led to the development of many drug resistant strains (Kwa *et al.*, 2007; Alekshun and Levy, 2007), which constitutes a major problem worldwide as the existing drugs are becoming ineffective to control them. Consequently, there is an urgent need to look for alternatives of synthetic antibiotics and other drugs. In recent years, much emphasis has been given to search new classes of drugs to which bacteria and fungi could not develop resistance. Due to high amount of diversified flora and diversified phyto-constituents, plants are found to be better source to search for new drugs. Numerous compounds that confer resistance to pathogenic bacteria, fungi and pests have been identified in plants, among them proteins and peptides with anti-microbial activity are important (Broekaert *et al.*, 1997; García-Olmedo *et al.*, 2001; Acharya *et al.*, 2007; Ribeiro *et al.*, 2007; Dolezilkova *et al.*, 2007). Antimicrobial peptides (AMP) are considered as attractive,

alternative therapies and are basic element of novel new generation of drugs for treatment of bacterial, fungal, cancer and viral infections (Giuliani *et al.*, 2007; Hoskin and Ramamoorthy, 2008)

AMPs (up to 100 amino acid residues) were discovered from variety of prokaryotes and eukaryotes including mammals, amphibians, insects and plants (Broekaert *et al.*, 1997; Fritig *et al.*, 1998; Ganz and Lehrer, 1998; García-Olmedo *et al.*, 1998; Hancock and Lehrer, 1998; Brogden *et al.*, 2003; Bulet *et al.*, 2004). AMPs form an important and ancient mechanism of innate resistance providing rapid and metabolically inexpensive first line of defense against pathogens (Broekaert *et al.*, 1997; Tollin *et al.*, 2003; Sugiarto and Yu, 2004). Till now many AMP sequences from both vertebrates and invertebrates are known, detailed information about antimicrobial peptides could be found on the website (http:/www.bbcm.univ.trieste.it/ossi/search.htm). There are several excellent reviews describing AMPs structure and mode of action (Gallo *et al.*, 2002; Izadpanah and Gallo, 2005; Huang, 2006). The best-known proteins involved in plant defense are lectins, pathogenesis-related proteins, hydroxyproline-rich glycoproteins, cyclophilin-like proteins, ribosome-inactivating proteins and protease inhibitors (Koiwa *et al.*, 1997; Selitrennikoff, 2001).

AGPs belong to class of hydroxyproline rich glycoproteins of plant origin (Showalter, 2001; Classen *et al.*, 2005). Their immunomodulatory potential is well established. The immunomodulatroy activity of a number of medicinal plants preparation reported to be due to their AGPs constituents (Classen *et al.*, 2006; Singha *et al.*, 2003; 2007). However, there are not many reports regarding its direct antimicrobial activity. Recently, Singha *et al.*, (2003) and Kottakis *et al.*, (2007) have reported that the AGPs have antibacterial and antifungal activities against various

pathogenic bacteria and fungi. The aim of the present study is to evaluate the antimicrobial activity of 46 kDa AGP purified from groundnut seedling. The findings are presented and discussed.

## 6.2 Materials and Methods

### 6.2.1 Microorganisms and growth conditions

The microorganisms used in this study were *Serratia marcescens* (MTCC 2296), *Bacillus subtilis* (MTCC 2423), *Staphylococcus aureus* (MTCC 2940), *Pseudomonas putida* (MTCC 2453), *Aspergillus niger* (ITCC 5454), *Candida albicans* (MTCC 227) *Microsporum gypseum* (ITCC 5277). All bacterial and fungal strains were obtained from Institute of Microbial Technology, Chandigarh, India. For routine maintenance the bacteria were grown on nutrient agar plate at 37 °C and fungi on potato dextrose agar (PDA) plate at 27 °C for 24 and 48 hr, respectively and were stored at 4 °C. *C. albicans* was routinely grown and maintained over YPD (0.5% yeast extract, 1% peptone, 2% dextrose) media. For experimental purposes the bacteria were inoculated in nutrient broth and were grown in control condition in an incubator shaker at 37 °C and 120 rpm up to mid log phase and fungi were inoculated in potato dextrose broth and grown at 27 °C, 120 rpm. All experiments of *C. albicans* were performed in YPD medium.

### 6.2.2 Antimicrobial activity assay

The antimicrobial assay was performed by disc diffusion method as described (Dahot, 1999), with slight modification. Mid log phase grown cultures of respective bacteria were used for testing antibacterial activity. Briefly, nutrient agar containing 1.5 % agar was prepared, autoclaved and allowed to cool down. A small volume (1x $10^6$ cells) of freshly grown respective bacterial culture was added aseptically to agar containing medium when its temperature was around 45 °C, mixed well and

poured immediately in to sterilized Petri plates. After the media was solidified 5 wells of 6 mm diameter each, were cut in each plate using sterilized steel cork borer. Different amounts of crude protein fraction viz. 20, 40, 60 and 80 µg were added to respective wells. Similarly the antimicrobial activity of purified 46 kDa AGP was also performed and 20, 40, 60 and 80 µg were used in respective wells. 50 mM Tris-HCl pH 8.0 was used as control in both assays. The plates were incubated at 37°C and formation of zone of inhibition around wells was recorded after 24 h.

Antifungal activity of crude protein fraction and purified AGP was tested according to the method reported by Terras *et al.*, (1995). Briefly, spores from freshly grown PDA plates were scraped using sterilized loop and suspended in potato dextrose broth to get free spores. Potato dextrose broth containing 1.5 % agar was prepared, autoclaved and allowed to cool down to 45 °C and to this 0.1 ml spore suspension (containing $1x10^4$ spores) of respective fungi was added, thoroughly mixed and poured immediately in sterilized Petri plates. After the media was solidified 5 wells of 6 mm diameter each, were cut using sterilized steel cork borer. Different amounts of crude protein fraction and purified AGP viz 20, 40, 60 and 80 µg were added to respective wells. 50 mM Tris-HCl pH 8.0 was used as control in both assays. The plates were incubated at 27 °C, for 48 h and formation of zone of inhibition around wells was recorded. Similarly, the assay was performed for *Candida albicans* but the medium was YPD.

### 6.2.3 Minimum inhibitory concentration and minimum bactericidal concentration determination

Minimum inhibitory concentration (MIC) is usually defined as the lowest concentration of drug that could inhibit the visible growth of organisms after overnight incubation (the incubation time may be more for slow growing organisms). MIC of crude protein and purified AGP for various bacteria used in the study has

been performed by broth dilution method. Briefly, mid log phase grown culture of each bacterium was prepared in nutrient broth. The cultures were diluted to give 1 x $10^6$ CFU/ ml, and 1 ml of these cultures of each bacterium was put in 15 different labeled tubes. Different amounts of crude and purified AGP (1-15 µg) in increasing order were added to respective culture containing tubes. All the tubes were incubated at 37 °C for 24 h in an incubator shaker. Media alone was used as control in each case. After incubation the MIC was determined. The MIC of crude protein against fungi was performed similarly but the media used was potato dextrose broth for other fungi and YPD for Candida and incubation was at 27 °C. The minimum bactericidal concentration (MBC) was determined by measuring the number of CFU on nutrient agar plate. Just after determination of the MIC, 50 µl of sample from tubes that showed no growth were spread on nutrient plate and incubated at 37 °C for 24 h. MFC is the lowest concentration of protein at which CFU was found to be negative.

### 6.2.4 Antibacterial activity by time-killing curve

Time killing studies were performed according to Mangoni *et al.*, (2004). *S. marcescens*, *B. subtilis*, *S. aureus and P. putida* were grown at 37 °C in nutrient broth medium until absorbance at $\lambda_{590}$ reached one. The cells were centrifuged, washed and resuspended in 10 mM sodium phosphate buffer (pH 7.4). 1 x $10^6$ cells were incubated with different concentrations like 20, 40, 60 and 80 µg/ml of purified AGP, at 37 °C. Aliquots of 10 µl were withdrawn at different time intervals and spread on nutrient agar plates. After overnight incubation at 37 °C, the surviving bacteria, expressed as CFU (colony forming units), were counted. As controls protein solvent (50 mM Tris-HCl pH 8.0) was used.

### 6.2.5 Scanning electron microscopy

Exponential phase nutrient broth cultures of different bacteria were centrifuged at 5000 x g for 5 min. The pellets were washed with 10 mM phosphate buffer pH 7.4, and resuspended in PBS buffer pH-7.4, to obtain $1x10^6$ cells/ml. The 1 ml bacterial suspension ($1x10^6$ cells) containing 40, 60 and 80 µg of AGP respectively, were incubated at room temperature for 30 min. Each sample was spread on a poly (L-lysine)-coated glass slide (18 mm x 18 mm) to immobilize bacterial cells. Glass slides were incubated at 30 °C for 90 min. Slide immobilized cells were fixed with 2.5 % (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer pH-7.2, extensively washed with same buffer and dehydrated with a graded ethanol series. The samples were observed under scanning electron microscope (Philips XL 30CP, Austria).

## 6.3 Results

### 6.3.1 Antimicrobial activity of crude protein fraction and 46 kDa AGP

It is clear from the antimicrobial assay that both the crude protein fraction and purified 46 kDa AGP have antimicrobial activity. The results of the antimicrobial assay of crude protein fraction and purified 46 kDa AGP are shown in Table-6.1 & 6.2 and fig. 6.1, 6.2 and 6.3. Crude protein exhibited antimicrobial activity against all tested bacteria and fungi. On the other hand purified 46 kDa AGP only showed antibacterial activity but no antifungal activity. Significant antibacterial and antifungal activity was observed at 20 µg and it was increasing with higher protein concentration in both crude and purified AGP. It was further noticed that the antibacterial and antifungal activity of crude protein and antibacterial activity shown by the purifed 46 kDa AGP, found to be broad spectrum as it was observed against all tested bacteria and fungi and was against both Gram positive (*S. aureus, B. subtilis*) and negative (*P. putida, S. marcescens*) bacteria. The MIC of the crude protein found to be 3, 4, 3 and

3 µg/ml, for *S. marcescens*, *P. putida*, *S. aureus* and *B. subtilis,* respectively (table-6.1) and MIC against fungi found to be 5, 4 and 4 µg/ml, for *A. niger*, *C. albicans* and *M. gypseum*, respectively.

Similar to the crude protein, purified 46 kDa AGP also showed broad spectrum antibacterial activity against all bacteria tested but do not had any antifungal activity at the concentrations tested. The MIC of the purified AGP was 7, 8, 8 and 6 µg/ml for *S. aureus B. subtilis, S. marcescens*, *P. putida* respectively (table-6.2). The MBC values for *S. aureus B. subtilis, S. marcescens*, *P. putida* were 25, 30, 30, 35 µg/ml respectively.

Kill curve study was performed to determine the rate of decrease in CFU of different bacteria upon incubation with different concentrations of 46 kDa AGP. The kill curve is shown in fig- 6.4. At 80 µg/ml concentration of 46 kDa AGP all bacteria tested were showing very low CFU at 15 min incubation time. The kill curves clearly indicate that CFU of different bacteria tested was dependent on incubation time as well as concentration of 46 kDa AGP.

### 6.3.2 Scanning electron microscopy

Scanning electron microscopy is a commonly used method to study the effect of AMPs on cell-surface morphology of bacteria and fungi in order to get information about its mode of action. Scanning electron microscopy was performed on exponential phase culture cells of respective bacteria under control and AGP treated conditions. The 46 kDa AGP was found to have significant influence on the bacterial cell morphology of all bacteria tested in present study. The scanning electron micrographs of *S. marcescens*, *B. subtilis*, *S. aureus* and, *P. putida* are shown in Fig, 6.5, 6.6, 6.7 and 6.8 respectively. Untreated samples of each bacteria (Fig, 6.5A, 6.6A, 6.7A and 6.8A) appear smooth and in proper shape, but the samples of these

**Table – 6.1** Antimicrobial activity of crude protein extract isolated from *Arachis hypoagea*

| Microorganism | Diameter of Inhibition Zone (mm)[a] Concentration of crude Protein per well | | | | | |
|---|---|---|---|---|---|---|
| | 20 µg | 40 µg | 60 µg | 80 µg | Reff | MIC |
| Bacteria | | | | | | |
| | | | | | Gentamycin[b] | |
| *Serratia macescens* (MTCC2296) | 12 | 14 | 17 | 20 | 21 | 3 |
| *Pseudomonas putida* (MTCC2453) | 12 | 15 | 18 | 21 | 18 | 4 |
| *Staphylococcus aureus* (MTCC2940) | 13 | 16 | 18 | 21 | 22 | 3 |
| *Bacillus subtilis* (MTCC2423) | 12 | 14 | 17 | 19 | 21 | 3 |
| Fungi | | | | | | |
| | | | | | Flucanazole[b] | |
| *Aspergillus niger* (ITCC5454) | 11 | 12 | 13 | 15 | 22 | 5 |
| *Candida albicans* (MTCC 227) | 10 | 11 | 13 | 14 | 18 | 4 |
| *Microsporum gypseum* (ITCC5277) | 11 | 12 | 14 | 15 | 23 | 4 |

Diameter of zone of inhibition (mm) including disc diameter of 6mm

[a] Means in triplicate (µg/ml)

[b] 30 µg/disc

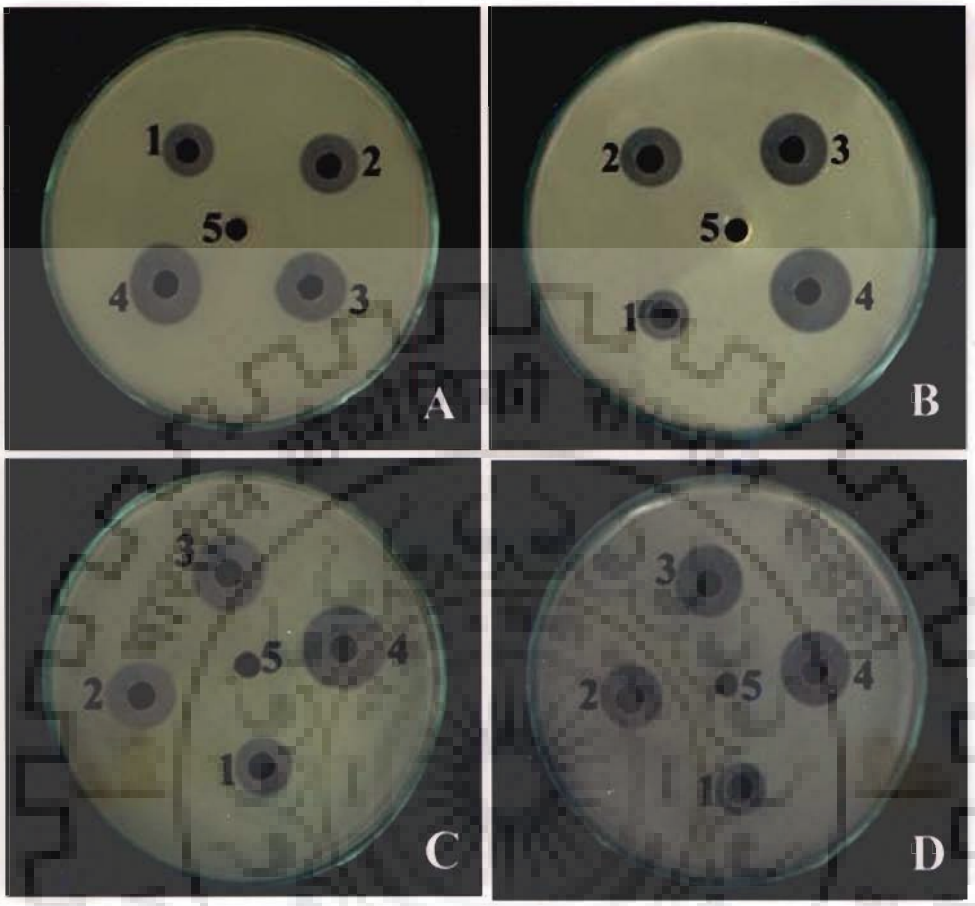



**Figure-6.1** Antibacterial activity of crude protein isolated from groundnut on bacteria by well diffusion method. Different bacteria tested were A- *S. marcescens*, B- *P. putida*, C- *S. aureus* & D- *B. subtilis*. Different concentrations of crude protein tested were 1- 20 µg, 2- 40 µg, 3-60 µg, 4-80 µg & 5- control. Formation of zone of inhibition was measured after 24 h.

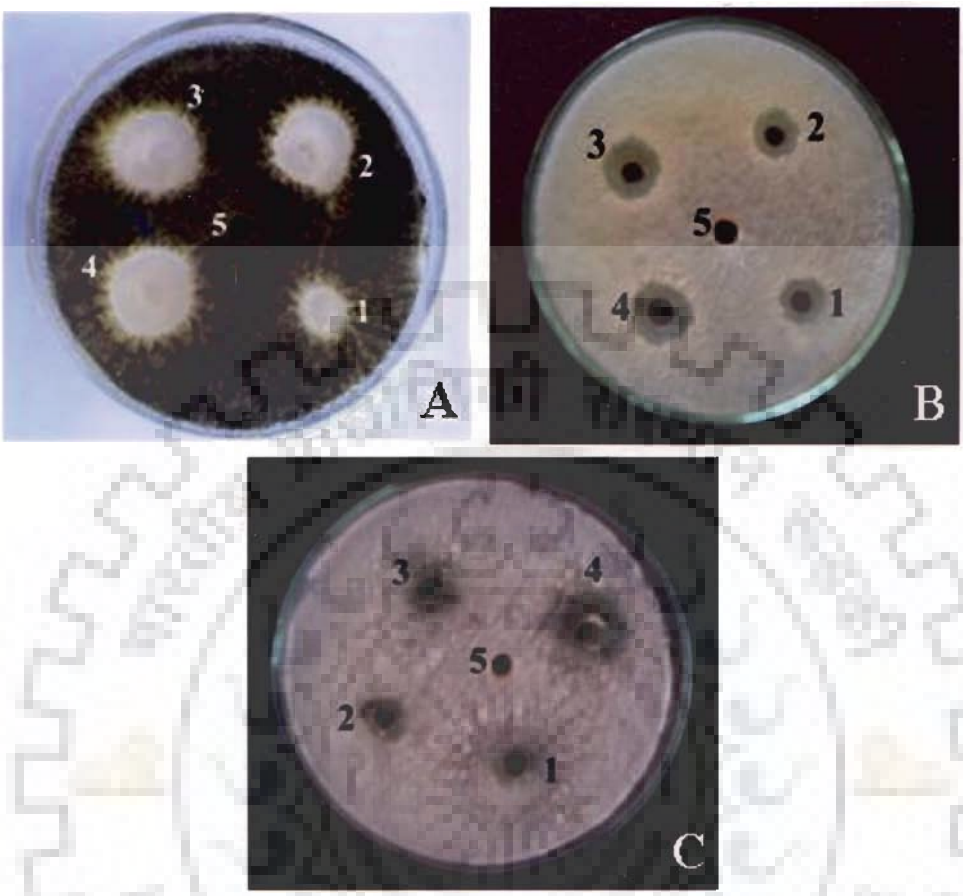



**Figure-6.2** Antifungal activity of crude protein isolated from groundnut on fungi by well diffusion method. Different fungi tested were A- *A. niger*, B- *C. albicans* & C- *M. gypseum*. Different concentrations of crude protein tested were 1- 20 µg, 2- 40 µg, 3- 60 µg, 4-80 µg & 5- control. Formation of zone of inhibition was measured after 48 h.

**Table – 6.2** Antimicrobial activity, MIC and MBC of 46 kDa AGP.

| Microorganism | Diameter of Inhibition Zone (mm)[a] Concentration of crude Protein per well | | | | | | |
|---|---|---|---|---|---|---|---|
| | 20 µg | 40 µg | 60 µg | 80 µg | Reff | MIC | MBC |
| Bacteria | | | | | (Genta)[b] | | |
| *Staphylococcus aureus* (MTCC2940) | 8 | 10 | 12 | 14 | 22 | 7 | 25 |
| *Bacillus subtilis* (MTCC2423) | 9 | 10 | 12 | 13 | 21 | 8 | 30 |
| Serratia marcescens (MTCC2296) | 7 | 8 | 10 | 11 | 21 | 8 | 30 |
| *Pseudomonas putida* (MTCC2453) | 9 | 10 | 11 | 13 | 18 | 6 | 35 |

Diameter of zone of inhibition (mm) including disc diameter of 6mm
[a] Means in triplicate (µg/ml)
[b] Gentamycin, 30 µg/disc

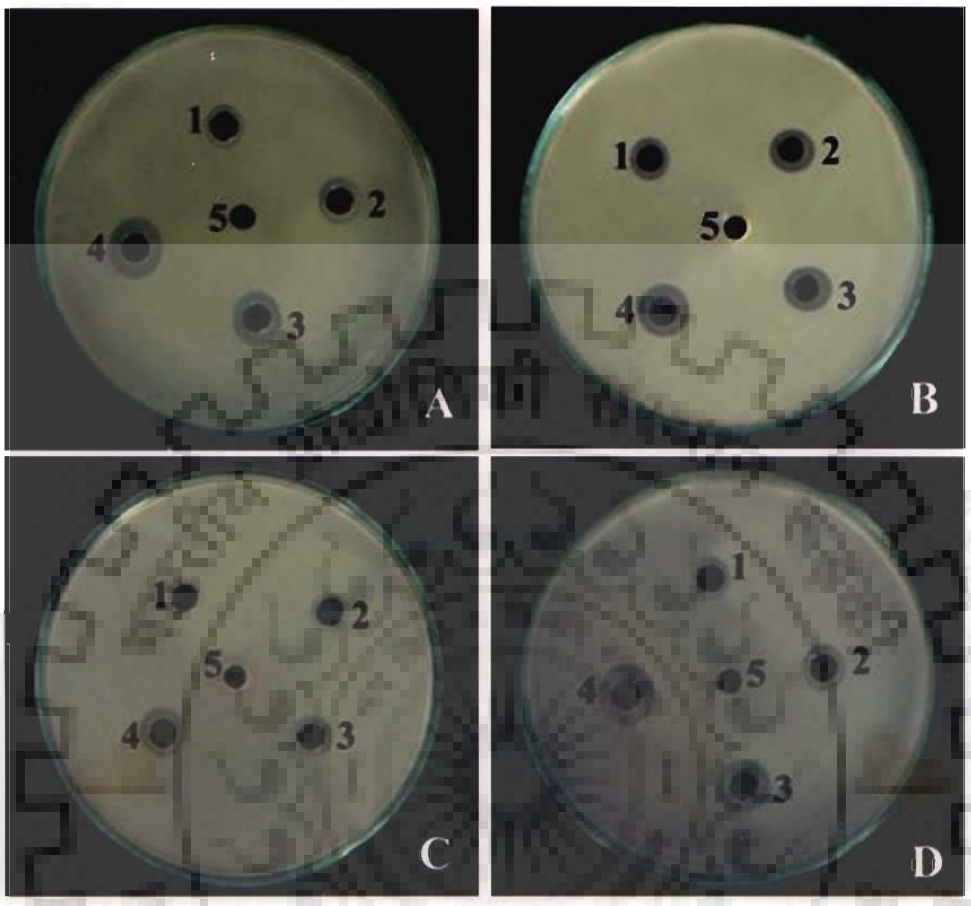



**Figure-6.3** Antibacterial activity of 46 kDa AGP isolated from groundnut on bacteria. Different bacteria tested were A- *S. marcescens*, B- *P. putida*, C- *S. aureus* & D- *B. subtilis*. Different concentrations of 46 kDa AGP tested were 1- 20 µg, 2- 40 µg, 3-60 µg, 4-80 µg & 5-control. Formation of zone of inhibition was measured after 24 h.

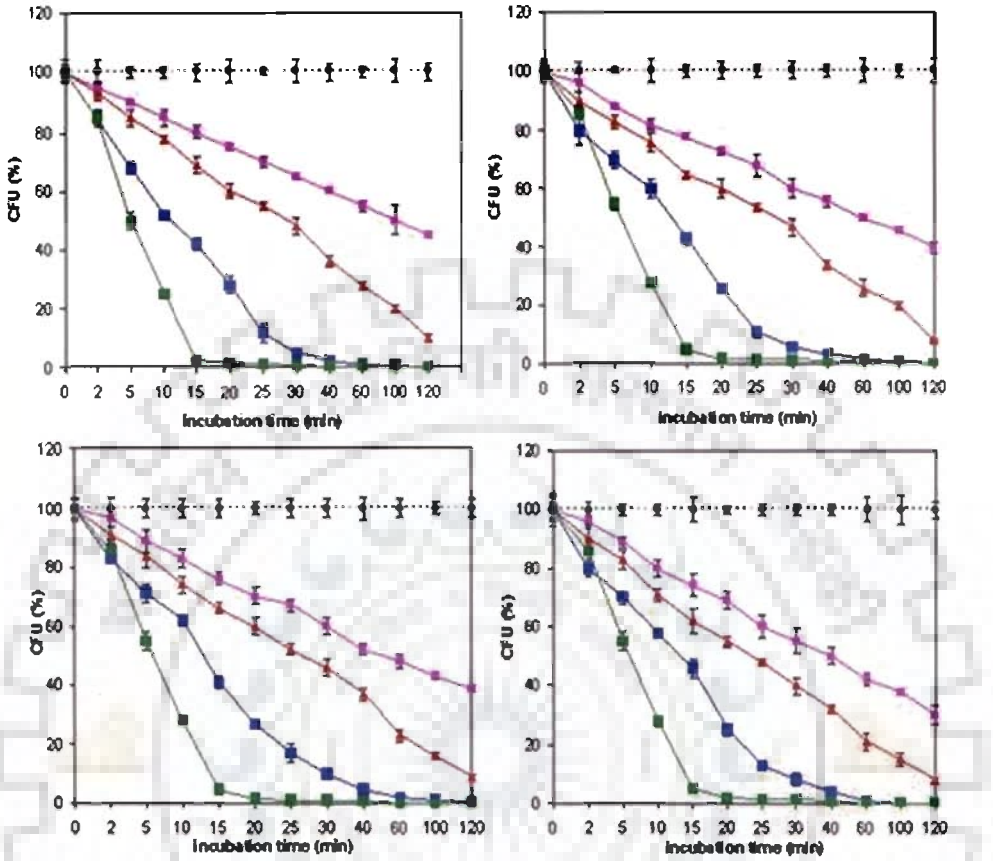



**Figure- 6.4** Time kill curve studies of bacteria. *S. marcescens* (a), *S. aureus* (b), *P. putida* (c) and *B. substilis* (d). All bacteria were grown in nutrient broth at 37 °C. Cells (1×106) diluted in 10 mM sodium phosphate buffer (pH 7.4) were incubated with 20 µg/ml (—•—), 40 µg/ml (—▲—), 60 µg/ml (—■—) and 80 µg/ml (—■—) of 46 kDa AGP at 30 °C. The number of surviving cells, at different incubation times, is expressed as the percentage of total cells. The control (···•···) is bacteria without protein. The values are means for three independent measurements.

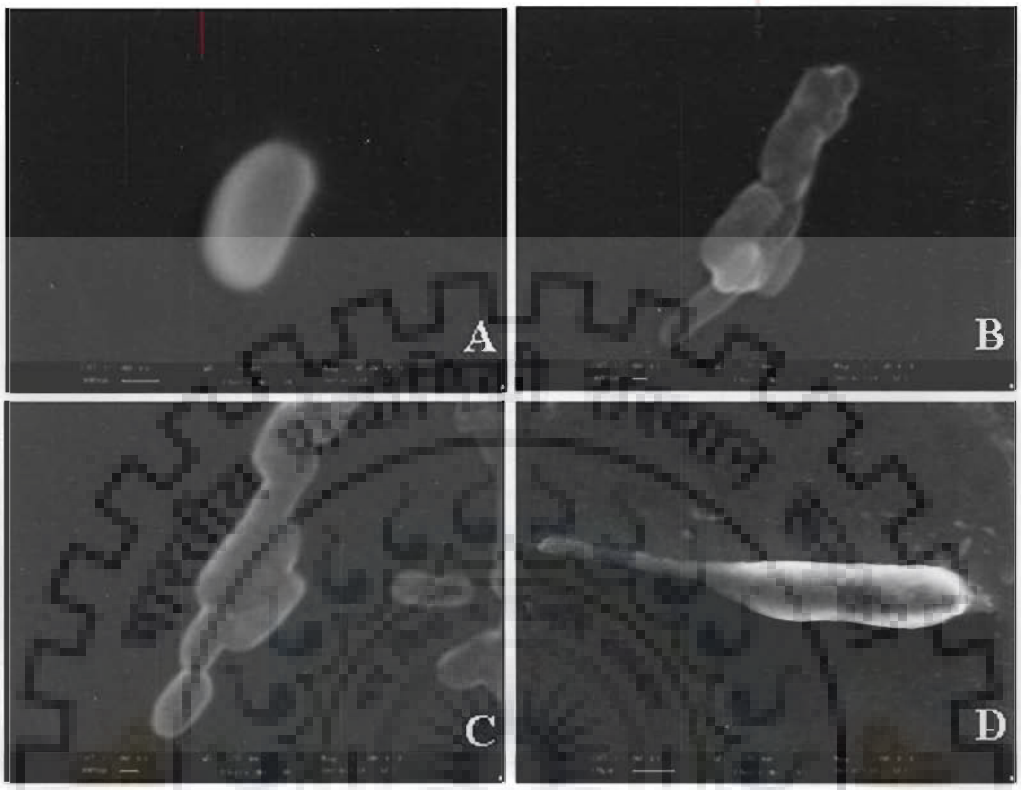



**Figure-6.5** Scanning electron micrograph of *S. marcescens* of untreated and treated cells with different concentrations of 46 kDa AGP for 30 min at room temperature. A- Control, B- 40 µg/ml, C-60 µg/ml & D-80 µg/ml. Change in cell morphology and deformation of surface could be easily seen.

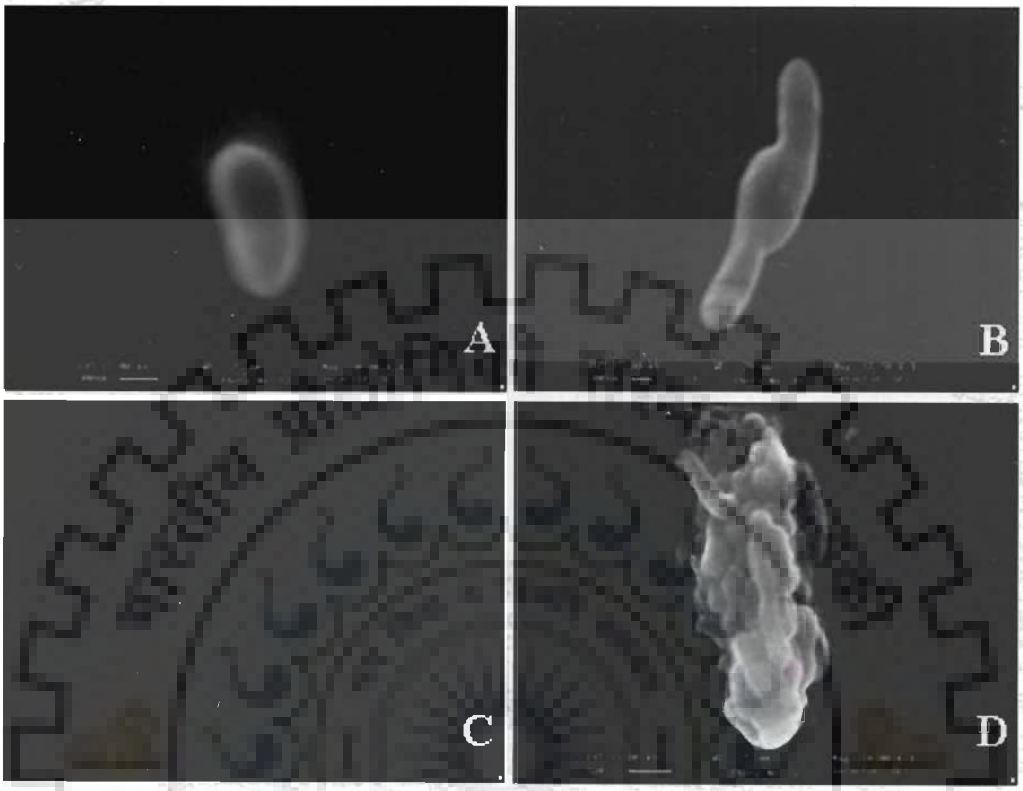



**Figure-6.6** Scanning electron micrograph of *B. subtilis* of untreated and treated cells with different concentrations of 46 kDa AGP for 30 min at room temperature. A- Control, B- 40 µg/ml, C-60 µg/ml & D-80 µg/ml. Change in cell morphology and deformation of surface could be easily seen.

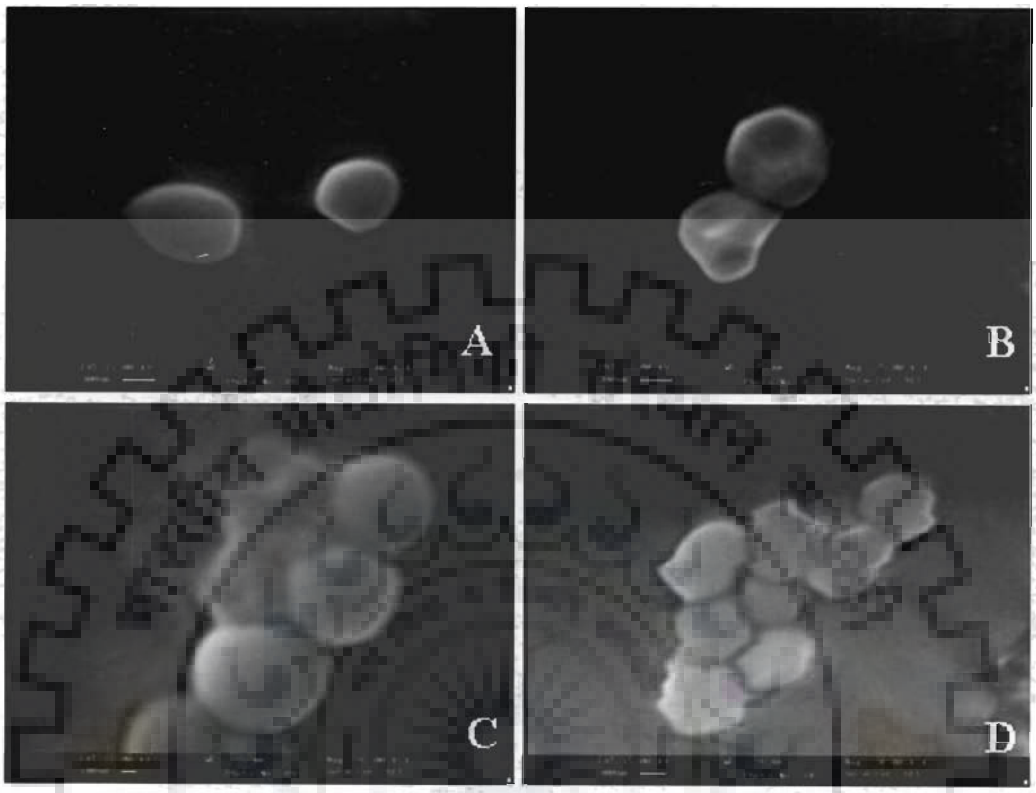



**Figure-6.7** Scanning electron micrograph of *S. aureus* of untreated and treated cells with different concentrations of 46 kDa AGP for 30 min at room temperature. A- Control, B- 40 µg/ml, C-60 µg/ml & D-80 µg/ml. Change in cell morphology and deformation of surface could be easily seen.

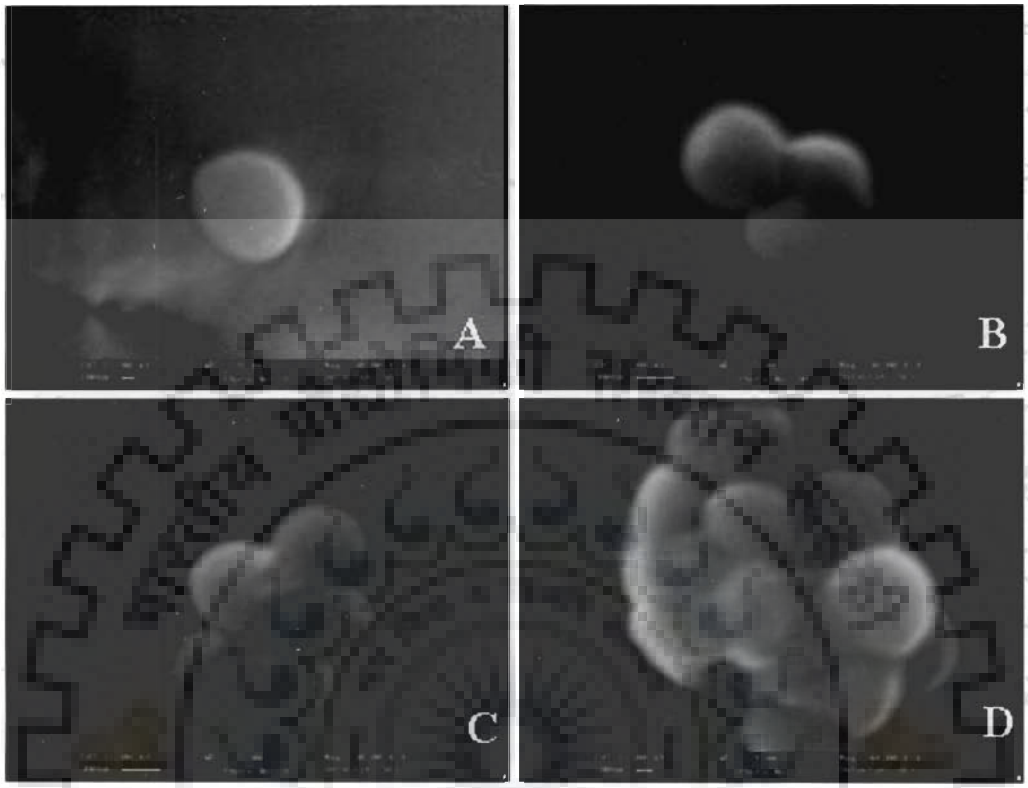



**Figure-6.8** Scanning electron micrograph of *P. putida* of untreated and treated cells with different concentrations of 46 kDa AGP for 30 min at room temperature. A- Control, B- 40 µg/ml, C-60 µg/ml & D-80 µg/ml. Change in cell morphology and deformation of surface could be easily seen.

bacteria treated with sub-lethal and lethal concentration 40, 60 and 80µg/ml of AGP for 30 min at room temperature, showed deformity on surface. It appeared rough and shape of the cell was changed as well it has become more elongated (Fig 6.5 B & C, 6.6 B & C, 6.7 B & C and 6.8 B & C). The cells were fully damaged at lethal concentration i.e 80 µg/ml (Fig 6.5D, 6.6D, 6.7D and 6.8D). The protein was found to cause merging of cell membrane in all the bacteria tested. A progressive bacterial cell membrane merging was observed with higher protein concentrations of 46 kDa AGP.

## 6.4 Discussion

Both crude and purified 46 kDa AGP showed broad spectrum antibacterial activity. However, crude protein also showed antifungal activity but 46 kDa AGP does not. Besides, there was slight difference in the MIC value of crude and purified 46 kDa AGP. It seems that the purified 46 kDa AGP in the present study did not have antifungal activity and the observed antifungal activity in crude protein fraction was due to presence of other non-AGP proteins. It is logical as the crude fraction was found to have a number of proteins as revealed from Coomassie blue gel staining. The loss of antifungal activity in purified 46 kDa AGP was due to removal of the other protein responsible for antifungal activity.

The MIC values of the crude and purified 46 kDa AGP were found to be significant compared to reference and were much lower than the MIC (25-100 µg/µl) of the various other antibacterial and antifungal proteins / peptides reported in earlier studies (Lear *et al.*, 1988; Andreu *et al.*, 1989). Compared to some earlier reported antimicrobial proteins/peptides where antimicrobial activity was found either against Gram positive or Gram negative bacteria, the antimicrobial activity of the 46 kDa AGP in present study was found to be against both Gram positive (*S. aureus, B. subtilis*) and negative (*P. putida*, *S. marcescens*) bacteria. The present study strongly

supports the earlier studies where it has been shown that the AGP have antimicrobial activity against some important pathogenic bacteria like *Helicobacter pylori* (Kottakis *et al.*, 2007). However, this is first report of isolation of 46 kDa AGP from groundnut with antimicrobial activity against both gram –ve and +ve bacteria.

Due to their important role in protection against infections, the search for AMPs have been one of the thrust areas of research globally and a large number of AMP have been isolated and characterized from diversified sources (Ngai and Ng, 2004; Park *et al.*, 2007; Ribeiro *et al.*, 2007; Wang *et al.*, 2007). Extensive studies have been performed revealing their structure and mode of action which has been described in detail in several recent reviews (Hancock and Chapple, 1999; Reddy *et al.*, 2004; Jenssen *et al.*, 2006). Although antimicrobial proteins and peptides differ significantly in their sequences, most of them share some similar features, such as a net positive charge and potential to adopt an amphipathic α-helix or β-sheet structure on their interaction with membranes. Studies on their mode of action have provided strong evidences that a common step in the killing mechanism is their interaction with bacterial plasma membrane (Oren and Shai, 1998; Cudic and Otvos, 2002). However, it is not clear whether the actual killing of bacteria is the result of one of the following factors: either peptide induces membrane permeation via pore formation or via membrane disintegration (Ludtke *et al.*, 1996; Shai, 1999) or their binding to cellular DNA (Tossi *et al.*, 2000) and interference with DNA and protein synthetic machinery (Subbalakshmi and Sitaram, 1998) or their binding to other intra cellular targets and thus inhibiting various vital functions (Salzet, 2002).

SEM has been used in majority of the study to see the cell morphology change and mode of action of AMPs (Mangoni *et al.*, 2004). SEM observations of the present study were similar to the earlier observations where antimicrobial protein and

peptides induced alterations on the surface morphology of bacterial and fungal cells. (Klainner *et al.*, 1970a, b). Although the exact mode of action of the AGP is not clear, it is very obvious that AGP has caused some activity on the bacterial cell surface that has resulted in morphological abnormality and ultimately its killing. The AGP treated cell became irregular and elongated and at higher concentration the cells were damaged completely. The cells were found to be aggregated due to their surface alterations while in some cell membranes were observed to be fully disintegrated resulting in cell lysis. These observations support the earlier SEM observations.

In conclusion, this study indicated that 46 kDa AGP has antibacterial activity and it is more likely that it causes some modulation at bacterial cell-surface which leads to cell lysis. Besides, it provides evidence that AGP have antimicrobial activity in addition to it is showing well known immunomodulatory activity. This is the first report of isolation of 46 kDa AGP from groundnut and showing its broad-spectrum antimicrobial activity. However, its actual mode of action is still need to be further investigated.

# PART-E

## 46 kDa AGP AS DRUG CARRIER

### 7.1 Introduction

In past few years, there has been alarming increase in life threatening mycotic infections in humans caused by various opportunistic fungi, mainly in immunocompromised hosts, such as cancer patients who have gone for surgery and AIDS patients (Bodey, 1977; Denning, 1991; Groll and Walsh, 2001). Chemotherapy has been the common strategy and a large number of antifungal drugs are being used to control the fungal infections. Among them the azoles and its derivatives are found to be the most effective and predominant (Johnson and Perfect, 2003; Donnelly and de Pauw, 2004). However, the fungal infection represents a major therapeutic challenge owing to the increased prevalence of organisms resistant to commonly used azoles (Singh, 2001; Ostrosky-Zeichner *et al.*, 2003). This fact poses severe limitation in their use besides the relatively high cost of azoles and its derivatives. Developing novel drugs and/or treatment strategies to fight these infections are therefore critical and this has led to the development of azoles with enlarged spectrum and to the discovery of other novel, broad-spectrum fungicidal drugs. Among the non-azoles drugs, amphotericin B (AmB) and its derivatives are the drugs of choice for the treatment of mycotic infection caused by wide range of fungi (Gallis *et al.*, 1990). In contrast to azoles, AmB is inexpensive while being highly fungicidal against most pathogenic fungi, and also found to be free of clinically meaningful resistance so far (Barrett *et al.*, 2003; Ostrosky-Zeichner *et al.*, 2003). The use of AmB, however, is hampered due to two main factors. The first is its dose related toxicity, mainly to the kidneys, central nervous system and liver, the frequency of which may be very high

(Sabra and Branch, 1990; Razzaque *et al.*, 2001) and its side effects, such as nausea, fever and shivering (Maddux and Barriere, 1980). Secondly, lack of solubility in injectable aqueous media due to its high hydrophobic nature (Hartsel and Bolard, 1996; Lewis and Wiederhold, 2003).

Several strategies have been developed and are in practice to circumvent the disadvantage of water insolubility and toxicities, such as AmB administration as a micellar dispersion in sodium deoxycholate (Cleary *et al.*, 2003; Clemons and Stevens, 2004), or as lipid formulation, such as liposomes (Adler-Moore and Proffitt, 2002; Manosroi *et al.*, 2004). These strategies, however, also have their limitations that include a narrow therapeutic index for micellar dispersions (Maddux and Barriere, 1980; Harbarth *et al.*, 2002), or high cost for lipid formulations (Cleary *et al.*, 2003). The development of other types of water-stable and well-dispersed aqueous solutions of AmB with low intrinsic toxicity and low manufacturing prices remains therefore highly desirable. A number of attempts have been made to design a water-soluble, injectable and stable formulation of AmB. These included the synthesis of *N*-acyl derivatives (Kobayashi *et al.*, 1985), *N*-methyl-*N*-d-fructosyl methyl esters (Szlinder-Richert *et al.*, 2004), the entrapment of AmB in amphiphilic micelles (Yu *et al.*, 1998a, b), or its association with microemulsions and monoglyceride–water systems (Moreno *et al.*, 2001). One of the approaches for improving drug performance and reducing toxicity is conjugation to a polymeric carrier (Domb *et al.*, 1996). In recent past, the AmB conjugation with polyethyleneglycol (Conover *et al.*, 2003) or arabinogalactan (Falk *et al.*, 1999) or polyvinylpyrrolidone (Charvalos *et al.*, 2006) respectively, have generated highly water-soluble conjugates that are found to be much safer and effective than the commonly used AmB-DOC formulation. The

glycoconjugates mainly the polysaccharides and polysaccharide-protein complexes could be a suitable alternative due to their high water solubility.

AGPs are a family of plant derived glycoconjugate with established history of pharmaceutical and other industrial applications, such as emulsifier, etc. (Egert and Beuscher, 1992; Hauer and Anderer, 1993; Yu *et al.*, 1998a, b; Classen *et al.*, 2000). AGPs isolated from a number of crops and medicinal plants have been purified and its structure have been determined (Gasper *et al.*, 2001; Showalter, 2001). Both the intact AGP and its purified polysaccharide fraction arabinogalactan are found to be highly water-soluble and possess a high degree of biocompatibility and are used in a number of pharmaceutical and neutraceutical preparations (Egert and Beuscher, 1992; Yu *et al.*, 1998a, b; Kelly, 1999; Classen *et al.*, 2000). In the present arena, many developments have been made to improve drug delivery and therapies (Chen *et al.*, 2006; 2007c; Hilder and Hill, 2008). AGPs can also serve as drug carrier because of its high water-solubility and high biocompatibility. In one of the earlier study, a water-soluble injectable conjugate of amphoptericin B-arabinogalactan (AmB–AG) was formed using commercial preparation of AmB and AG (Falk *et al.*, 1999). The conjugate was found to be stable and shown to increase the solubility and stability of AmB in aqueous solution. Conjugation significantly reduces AmB toxicity and poses high degree of biocompatibility (Ehrenfreund-Kleinman *et al.*, 2002; Folk *et al.*, 2004). Although the simple two-step Schiff base method has been established for conjugation of AmB with AG, yet no attempt had been made so far to conjugate the AmB with AGP. The aim of the present study was to reduce the toxicity of AmB by preparing a water-soluble injectable AGP–AmB conjugate without affecting its antifungal activity. The present work describes conjugation of AGP with AmB. The water solubility and toxicity of the conjugate was evaluated and its antifungal activity

was tested against clinical isolate of *Candida albicans*. The findings are presented and discussed.

## 7.2 Materials and Methods

### 7.2.1 Growth and maintenance of *C. albicans* clinical isolates

Seven clinical isolates of *C. albicans* were obtained from AIIMS, New Delhi, India. The clinical isolates were routinely grown and maintained in YPD (0.5% yeast extract, 1% peptone, 2% dextrose) medium in laboratory unless otherwise mentioned.

### 7.2.2 Purification and quantification of AGP

Purification and quantitative determination of AGP was performed as per method already described in previous section (3.2.6).

### 7.2.3 Conjugation of AGP with AmB

The conjugation of AGP to AmB was carried out by a Schiff base reaction as per protocol described (Falk *et al.*, 1999) with little modification. The AGP was first converted to an oxidized dialdehyde form (DAAGP), which was then conjugated to AmB by amine bond with the amino group (−NH2) of the AmB. To the AGP (1%) solution in water, potassium periodate (0.05 M) was mixed and the mixture was stirred at room temperature, until it was dissolved completely. This resulted in dialdehyde AGP formation (DAAGP), which was purified from excess perisodate using Dowex-1-acetate (Sigma, USA) column (6mm×8 mm). The purified DAAGP formed was resuspended in 0.2 M borate buffer pH 11.0 (12.5 mg/ml) and mixed with AmB (MERCK, Germany) final concentration of 6.25 mg/ml. The conjugation reaction was carried out at 37 °C for 48 h. After incubation 1.2 M sodium borohydrate was added to the conjugation mixture at 4 °C for 60 min with stirring. The final conjugate was purified by dialysis against deionized water for 48 h at 4 °C. The dialysate was centrifuged at 2000×*g* for 10 min and lyophilized. The conjugate was

stored in dry powdered form. The conjugate was filter sterilized through 0.2 μm pore size membrane prior to use.

### 7.2.4 Susceptibility using broth method

The drug susceptibility testing was done against seven *C. albicans* clinical isolates. MICs were determined by microdilution broth method as per standard norms. A 10 mg/ml stock solution of AmB and AGP–AmB was prepared in dimethyl sulphoxide (DMSO) and water, respectively. Various serial dilutions of both stocks (ranging from 120 to 0.01 mg/ml) were prepared using filter sterilized RPMI 1640 broth medium, pH 7.0 (Hi Media India). In 96 well round bottom microtiter plate 0.1 ml of various serial dilution of drugs were taken in triplicate. Pre-inoculum of *Candida* isolates were prepared in YPD medium.

From the pre-inoculum, the final inoculum of each isolate was prepared using sterile RPMI 1640 broth to give a $10^5$ cells/ml, which was determined by counting using hematocytometer. To the microtiter plate wells containing 0.1 ml of serially diluted drugs, 0.1 ml of each inoculum was added. For control 0.1 ml of drug free medium and 0.1 ml of inoculum were taken. The experiment was carried out in triplicate. The microtiter plates were incubated at 35 °C for 24 h. The growth in each well was determined visually. The MIC was defined as lowest drug concentration that results in complete inhibition of visible growth. The minimum fungicidal concentration (MFC) was determined by measuring the number of CFU on YPD plate. Just after determination of the MIC, 50 μl of sample from wells that showed no growth were spread on YPD plate and incubated at 35 °C for 24 h. The MFC was determined as lowest concentration of drug at which CFU was found to be negative.

### 7.2.5 Susceptibility using disc method

Drugs susceptibility was also tested against various *C. albicans* isolate. The *Candida* cells ($10^4$/ml) were mixed with sterile YPD medium and poured into petri dishes. The plates were allowed to solidify. After solidification sterile filtered disc were put on plate and loaded with different concentrations of AmB and AGP–AmB conjugate. The plates were incubated for 24 h at 30 °C and formation of halo zone around disc was monitored.

### 7.2.6 *In vitro* toxicity assay

*In vitro* toxicity was studied using sheep erythrocytes (SRBCs). SRBCs were suspended in phosphate buffer saline and washed twice by centrifugation at 3000×*g* for 10 min. 0.1 ml of serially diluted drugs were taken in glass tubes and mixed with 0.9 ml of SRBCs. The tubes were incubated for 1 h at 37 °C. The extent of hemolysis was measured visually.

### 7.2.7 Toxicity in animal model

*In vivo* toxicity was tested using albino BALB/C mice as described (Falk *et al.*, 1999). Male BALB/C mice approximately 25 g weight was injected via tail vein with various doses of AmB and AGP–AmB conjugate. Each dose was given as a single bolus injection of 0.1 ml intra-venously. For each dose 10 mice were injected after every 10 min until death was observed. The survival of the mice that received the maximum tolerance dose (MTD) was monitored for 8 days.

## 7.3 Results

### 7.3.1 Conjugation of AGP and AmB

Conjugation of AGP and AmB was carried out as described in fig.7.1. Since the yield of conjugation depend on both concentration and pH of reaction as indicated in earlier studies. The conjugation of AGP and AmB was done at pH 11.0 at which

maximum yield were obtained. The general scheme of conjugation reaction is explained in fig. 7.1. In general, the conjugation is two-step process, firstly the AGP is converted to an oxidized dialdehyde form, which was purified and then reacted with AmB and finally the AGP–AmB conjugate was formed by reaction of –NH2 group of AmB with dialdehyde AGP. Conjugation product was confirmed by presence of AmB in the final product by measuring absorbance at 250–500 nm and its reaction with Yariv reagent (fig. 3.1, chapter 3, part A).

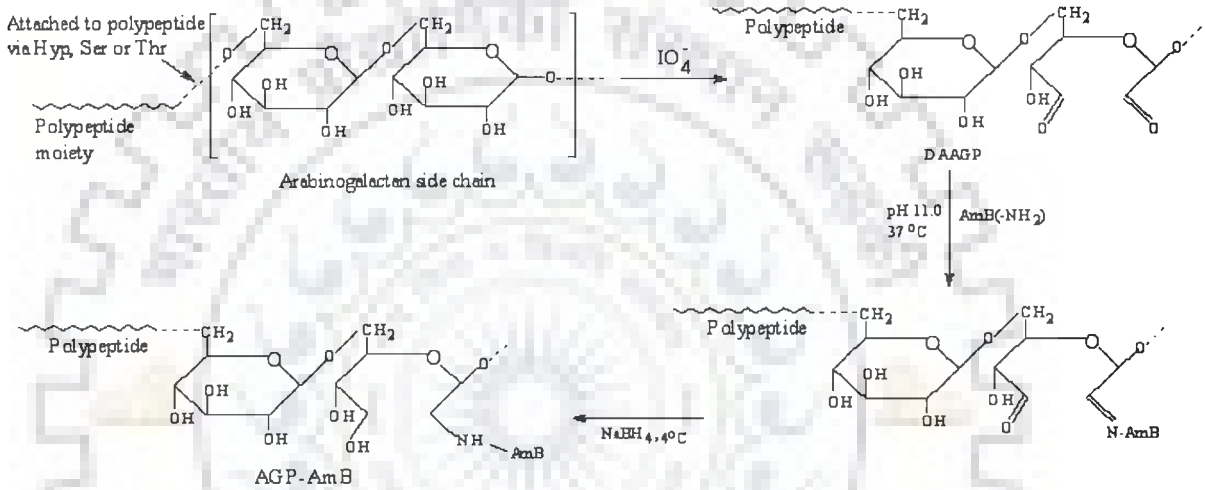



**Figure- 7.1** General scheme of AGP and AmB conjugate preparation. This is a two-steps process where in AGP is first converted to dialdehyde form (DAAGP) by treatment with periodate. The DAAGP after purification, is then mixed with AmB and AGP-AmB conjugate formed via amide linkage using –NH2 of AmB. (**AGP-** a hypothetical general structure of Arabinogalactan protein, where arabinogalactan side chain is attached to polypeptide through Hyp, Ser or Thr. **DAAGP-** dialdehyde form of AGP; **AGP-AmB-** arabinogalactan protein-amphotericin conjugate).

### 7.3.2 Evaluation of antifungal activity of the AGP–AmB conjugate

The antifungal activity of the AGP–AmB conjugate against seven *C. albicans* clinical isolates was determined as described in materials and methods section. The MICs and MFCs are given in Table 7.1. The AGP–AmB conjugate showed almost similar antifungal activity as free AmB. Thus, it is clear that AGP binding does not

reduce the antifungal activity of AmB. Similar results were also observed in disc method assay (fig. 7.2). The AGP–AmB conjugate and AmB showed almost similar antifungal activity against different *C. albicans* clinical isolates, except the isolate no. 7 which seems to be AmB resistant (fig. 7.2 C). Relatively higher MIC and MFC were observed in this isolate compared to others (Table 7.1, isolate no.7).

**Table 7.1 MICs & MFCs of AmB and AGP-AmB conjugate against various *Candida albicans* clinical isolates.**

| Name of Isolate | AmB | | AGP-AmB | |
|---|---|---|---|---|
| | MIC (μg/ml) | MFC (μg/ml) | MIC (μg/ml) | MFC (μg/ml) |
| *Candida albicans* isolate No. 1 | 0.14-0.30 | 0.35-0.50 | 0.14-0.30 | 0.35-0.50 |
| *Candida albicans* isolate No. 2 | 0.14-0.30 | 0.35-0.50 | 0.14-0.30 | 0.35-0.50 |
| *Candida albicans* isolate No. 3 | 0.14-0.35 | 0.35-0.50 | 0.14-0.35 | 0.35-0.50 |
| *Candida albicans* isolate No. 4 | 0.14-0.35 | 0.35-0.50 | 0.14-0.35 | 0.35-0.50 |
| *Candida albicans* isolate No. 5 | 0.14-0.30 | 0.30-0.50 | 0.14-0.35 | 0.30-0.50 |
| *Candida albicans* isolate No. 6 | 0.12-0.30 | 0.30-0.50 | 0.12-0.35 | 0.30-0.50 |
| *Candida albicans* isolate No. 7 | 0.20-0.50 | 0.50-1.20 | 0.20-0.50 | 0.50-1.20 |

AmB = Free Amphotericin B

AGP-AmB = Arabinogalactan protein-Amphotericin B conjugate

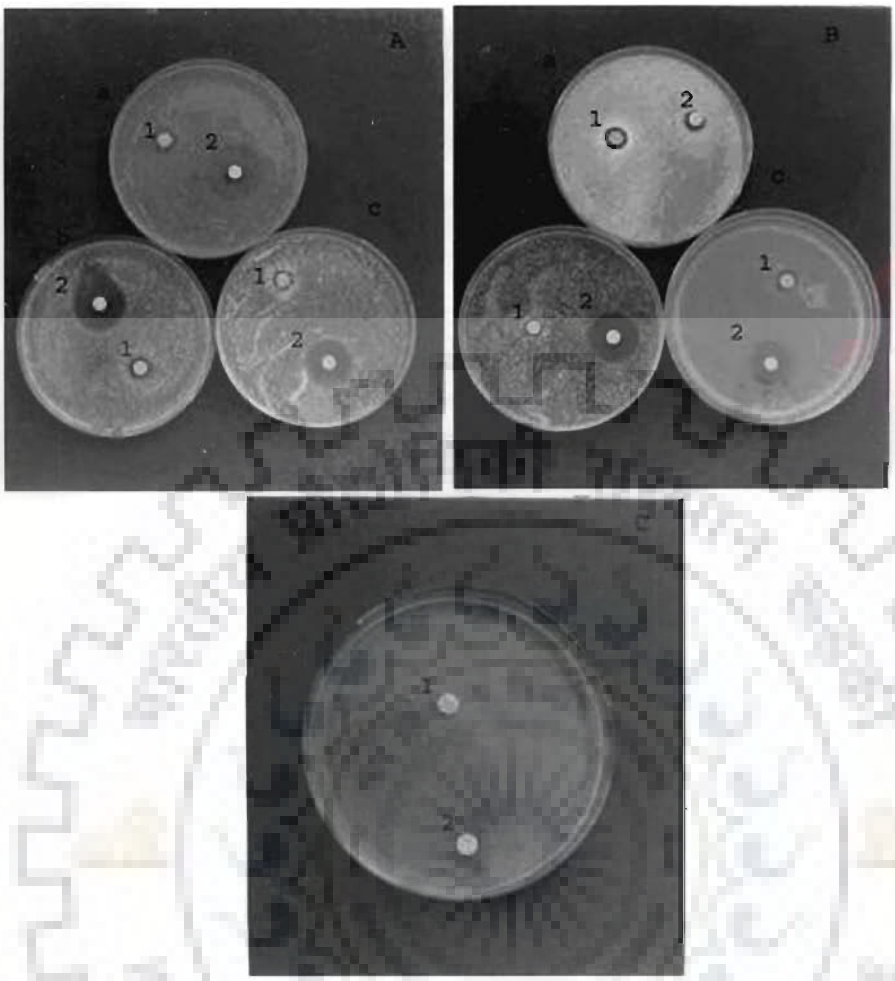



**Figure- 7.2** Antifungal activities of free AmB and AGP-AmB conjugate were tested against different *Candida albicans* clinical isolates by disc method. In total seven isolated were used however, results of only few are shown here. (A) Antifungal activity of AmB against clinical isolate no 1 (a), isolate no 2 (b) and isolate no 3 (c), respectively. 1 and 2 are two different concentrations 10 and 20 µl of AmB (1mg/ml), respectively. (B) Antifungal activity of AGP-AmB conjugate against clinical isolate no 1 (a), isolate no 2 (b) and isolate no 3 (c) respectively. 1 and 2 are two different concentrations 10 and 20 µl of AGP-AmB (1mg/ml), respectively. (C) Antifungal activity against an amphotericin B resistance isolate (isolated no 7), 1-AmB and 2-AGP-AmB, respectively.

### 7.3.3 *In vitro* and *in vivo* toxicity studies

*In vitro* toxicity was determined visually by hemolysis of SRBCs by AmB and AGP–AmB conjugate, respectively. No hemolytic activities were monitored up to 1.2 mg/ml of AGP–AmB conjugate. However, a very little hemolytic activity was observed at concentration >1.5 mg/ml, the highest concentration tested. On the other hand, hemolysis was observed at much lower concentration (10 μg/ml) of free AmB. *In vivo* toxicity was carried out in BALB/C mice. The result is shown in fig. 7.3. It is clear that AGP--AmB conjugate showed much higher MTD (45 mg/kg) compared to AmB, which is 5 mg/kg. This indicates that AGP–AmB conjugate is less toxic than AmB.

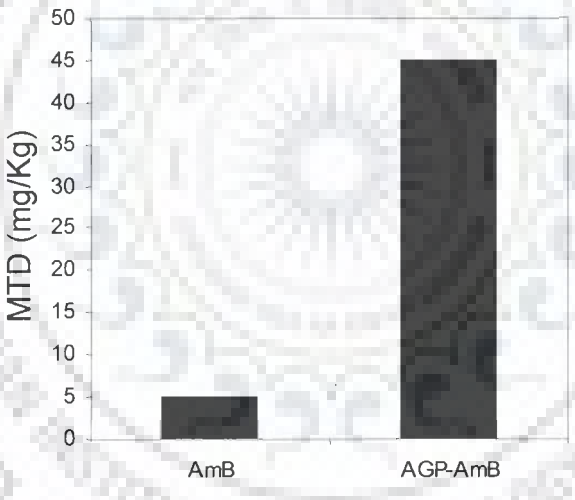



**Figure- 7.3** *In vivo* toxicity assay of AmB and AGP-AmB in mice. Maximum tolerance doses (MTD) were determined. AmB-amphotericin B, AGP-AmB- arabinogalactan protein-amphotericin conjugate.

## 7.4 Discussion

In present study, an attempt has been made to develop a new formulation of AmB that is AGP–AmB conjugate, which could be highly water-soluble and safer than the other commonly used water insoluble and liposomal formulations. The AGP

was chosen to form water-soluble AGP–AmB conjugate due to two main reasons. Firstly, it is closely related to AG and possesses all of its important properties, such as high water solubility, biodegradability, biocompatibility and ease of conjugation in aqueous media. Secondly, the relatively easy purification of AGP compared to AG which is more tedious. AGP from *A. hypogaea* was isolated and purified using Yariv reagent which is specific for AGP. The purity and quantitative estimation was done using Yariv reagent (fig. 3.1). Halo zone was observed with AGP preparation but not with non-AGP. The purified AGP was then conjugated with AmB under conditions, which results in maximum yield. The AGP–AmB conjugation was carried out in two steps. Firstly, the AGP was oxidized by reaction with the oxidizing agent potassium periodate. Since the AmB being a polyene is sensitive to oxidation, therefore, it was essential to prevent the oxidation and degradation of AmB by oxidizing agent. Thus, the excess iodate and periodate ions were removed from the oxidized AGP using ion exchange column before conjugation to AmB otherwise, it will lead to poor yield. In the second step, the oxidized AGP molecules were conjugated to AmB under optimum conditions. As it has been observed in one of the earlier study that conjugation of AG (the polysaccharide fraction) with AmB, was optimal at pH 11.0, in borate buffer (Falk *et al.*, 1999). Therefore, in order to achieve high yield in present study AGP–AmB conjugation was carried out in borate buffer, pH 11.0, at which maximum yield was expected. The conjugation under these conditions resulted in high yield (up to 90%). The high yield could be due to the fact that boric acid forms complex with AmB, which increases its water solubility and minimize aggregate formation, which is one of the major problem of AmB (Ernst *et al.*, 1981; Straus and Kral, 1982). The solubility of AmB-borate complex was found to be highest at pH 11 or above, while lesser at lower pH and minimum in neutral solution. The formation of

such complexes facilitates the AmB molecules to dissolve into reaction solution, and makes them available for conjugation with oxidized AGP. The synthesis of AGP–AmB conjugate was confirmed by measuring absorbance of the final product at wavelength range 250–500 nm using UV–visible spectrophotometer (Perkin-Elmer, Lambda Bio 40, USA) and also its reaction with Yariv reagent (fig. 3.1). A number of studies showed that the free AmB solution has typical peaks at 408, 385, 365 and 348 nm, which is concentration dependant and linked to aggregate formation (Lamy-Freund *et al.*, 1993; Balakrishan and Easwaran, 1993). The ratio *A*348/*A*408 is indicative of the aggregated/monomeric ratio and a value of ~0.25 being predominated by monomeric form, while the value >2.0 is predominated by aggregate form. Similar to the previous studies, free AmB solution has showed its characteristic peaks at mentioned wavelengths range. On the other hand, free AGP solution has peak at 250 nm, while the AGP–AmB conjugate solution showed a composite spectrum with typical peaks of both AmB and AGP. Unlike the free AmB, AGP–AmB conjugate has constant absorbance at all concentrations, which indicates that it stabilized the drug and reduced aggregate formation. However, in present work no further study was performed to confirm the aggregate formation. The final product showed composite spectra of both AmB and AGP, also reacted with Yariv reagent. Thus, both the spectroscopy and Yariv reagent confirmed the AGP–AmB conjugate formation.

The AGP–AmB conjugate have shown high water solubility (up to range of 85 mg/ml) and poor solubility in DMSO (1 mg/ml). On the other hand, AmB is poorly soluble in water (0.1 mg/ml at pH 2 and 11), and highly soluble in DMSO (up to 40 mg/ml) as observed in present study and also in earlier report (Budavari *et al.*, 1989). Thus, AGP–AmB conjugates showed improved quality compared to free AmB. The

antifungal activity and toxicity of the AGP–AmB conjugate were tested in *in vitro* and *in vivo* conditions. It was clear from the antifungal activity assays both by broth serial dilution (Table 7.1) as well as disc method (fig. 7.2), against several *C. albicans* clinical isolates that AmB and AGP–AmB conjugate have almost similar antifungal activity. These results show, quite unambiguously, that the activity of AmB was not impaired by its conjugation with AGP (based on MIC, MFC). Being a member of polyene antifungal, AmB involves the formation of complex with ergosterol leading to the creation of transmembrane channels that disrupt the membrane permeability properties of the fungal cells (Baginski *et al.*, 2002). This interaction relies on the availability of free AmB. Thus, it is clear that conjugation to AGP does not affect the interaction of AmB with ergosterol present in the fungal membrane. A similar observation is, nevertheless, made for the liposomal form of amphotericin (Adler-Moore and Proffitt, 2002) and AmB–polyvinylpyrrolidone (PVP) conjugates (Charvalos *et al.*, 2006). On the other hand, the *in vitro* and *in vivo* toxicity assays showed reduction in toxicity. It has been reported that besides its interaction with ergosterol, amphotericin may, however, also interact with cholesterol, which is the most likely explanation for its toxicity towards eukaryotic cells (Brajtburg and Bolard, 1996). Therefore, measurement of hemolytic index of SRBC is one of the parameter and is one of the methods commonly used for *in vitro* toxicity of AmB (Foster *et al.*, 1988). It was observed that hemolytic index of AGP–AmB conjugate was significantly higher than that of AmB (>1 mg/ml). The toxicity of the AmB–AGP complexes appeared markedly decreased compared with free AmB, based on the measurement of its lytic activity towards red blood cells. Amphotericin–cholesterol interaction occurs more easily when the drug is in an aggregated form as compared with monomers (Barwicz and Tancrede, 1997; Huang *et al.*, 2002). A reduction of

aggregation by complex formation with AGP could, therefore, have been critical to explain the lesser cytotoxicity of AGP–AmB compared with AmB. *In vivo* toxicity study in mice model also confirmed that AGP–AmB conjugate was significantly lesser toxic than free AmB, as MTD was 45 and 4 mg/kg, respectively (fig. 7.3). This could be due to the improved water solubility which leads to lower cellular accumulation of the drug as suggested in earlier studies (Folk *et al.*, 2004; Charvalos *et al.*, 2006). However, in present work, no study has been performed regarding cellular accumulation of drug. Thus, it is clear from the present work that conjugation of AGP with AmB significantly reduces its toxicity and does not have any adverse effect on its antifungal activity. Therefore, it is suggested that AGP could serve as novel potent carrier for AmB drug formulation. Study regarding the testing of the efficacy of conjugate in murine model of candidosis needs to be done to further confirm its potential. Since the AGPs from various sources found to have immuno-stimulatory activity and the fungal infections are more common in immuno compromised host, it is speculated that conjugation of AmB to AGP (with proven immuno-stimulatory activity) could be advantageous by having both antifungal as well as immuno-stimulatory properties. However, this is merely a speculation unless AmB is conjugated to AGP (with proven immuno-stimulatory activity) and the final conjugate is tested for its antifungal and immuno-stimulatory activity. Neither it was tested nor do claimed regarding immuno-stimulatory properties of AGP used in present study.

# Summary

AGPs are a group of proteins which contain both carbohydrate and protein as their constituents and are collectively known as Hydroxyproline rich glycoproteins. AGPs are widely distributed among the plant kingdom from bryophytes to angiosperms. Study of AGPs has been thrust area of research because of its important role in plant growth and development and its various commercial and therapeutic potential. AGPs from several important crops and medicinal plants have been isolated.

Groundnut (*Arachis hypogaea* L.) is one of the major oilseed crops and is a member of genus *Arachis* and family Leguminoceae. Groundnut is an important commodity in many developing countries for both direct human food and oil production, particularly in India where the nitrogen (N)-rich crop residues are also used as fodder. In addition, it is a good source of minerals like P, Ca, Mg and K. Seeds also contain vitamins E, K and B group. Although the AGPs have been identified as an important molecule from several economically important plants, yet no study regarding these has been carried out in *A. hypogaea.* Therefore, in the present study an attempt is being made to isolate the AGP from groundnut (*A. hypogaea)* seedling and explore its possible cellular functions and therapeutic potential.

**Isolation, purification of AGP from groundnut seedlings and its immunolocalization**

The study of AGPs in past have been relatively difficult task due to lack of precise procedure to get homogenous protein. But the discovery of an important class of molecular probes for the investigation of AGPs by Yariv *et al.*, (1967) has been a major breakthrough in AGP research. The general chemical name for this class of intensely red-colored probes is 1,3,5-tri-(p-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene. Although

the common names "Yariv antigens" and "Yariv reagents" have been widely used for these probes but the name "Yariv phenylglycosides" is more appropriate. The interaction of Yariv reagent with AGP is specific and among the numerous Yariv phenylglycosides only $(\beta\text{-D-Glc})_3$ and $(\beta\text{-D-Gal})_3$ Yariv phenylglycosides precipitate AGPs but the $(\alpha\text{-D-Gal})_3$ and $(\alpha\text{-D-Man})_3$ Yariv phenylglycosides do not.

Due to the specificity of $(\beta\text{-D-Glc})_3$ and $(\beta\text{-D-Gal})_3$ Yariv phenylglycoside for AGPs, these are commonly used for detection, purification and functional study of AGPs from various plants. In the present study, the $(\beta\text{-D-Gal})_3$ Yariv phenylglycoside was used for AGP purification. The proteins were extracted using the extraction conditions that facilitate AGP extraction and its reaction with Yariv reagent. The crude protein fraction had several proteins as revealed from coomassie blue stained gel, while the alcian blue stained gel showed relatively few glycoprotein in preparation. Since the alcian blue staining of the gel indicated the presence of glycoprotein in crude extract and the crude protein fraction reacted with Yariv reagent in gel diffusion assay, it ascertained that crude protein does contain AGPs. The AGP from crude extract was purified by Yariv reagent precipitation and a major AGP of 46 kDa was obtained. Though the protein was purified using Yariv reagent, its AGP nature was also confirmed by gel staining with Yariv reagent and also by its recognition with an AGP specific monoclonal antibody LM-2 (a kind gift from Dr. Paul Knox, University of Leeds, UK).

A macromolecule is considered as AGP if it has both carbohydrate and polypeptide components and if the carbohydrate component is rich in galactosyl and arabinosyl residues. Furthermore, the galactosyl residues in AGPs occur predominately in (1-3)-β-D-, (1, 6)-β-D- and (1,3,6)-β-D-galactopyranosyl linkage. These linkages are

characteristic of type-II arabinogalactans and distinguish AGP from other arabinogalactans found in plants and microorganisms which has predominately (1, 4)-β-D-galactopyranosyl linkage which do not react with Yariv reagent. Although in the present work no detailed study regarding sugar analysis has been performed. However, since this reacted with $(\beta\text{-D-Gal})_3$ Yariv phenylglycoside and also being recognized by AGP specific monoclonal antibody LM-2, therefore, it is indeed an AGP.

The AGPs are highly polymorphic molecules and there are variations in both protein and carbohydrate components among various AGPs. However, there is inconsistency in the nomenclature of AGPs. Depending upon the protein and sugar contents AGPs could be named as proteoglycans or glycoproteins. Most of the AGPs typically consist of about 10% protein and about 90% carbohydrate, the latter being present in the form of large polysaccharide chains and are usually named as proteoglycans. On the other hand, it should be noted that some AGPs or AGP-like molecules with unusually high protein contents in the range of 30% or even 65% have been reported, and these macromolecules are classified as glycoproteins. The AGP purified in the present study belongs to the later group, this is mainly because it has protein content in the range of 60% and sugar in the range of 40% as observed from protein and sugar estimation. This was also reflected from the relatively sharp or less diffused band in gel as observed by staining with coomasie blue and Yariv reagent. On the other hand, relatively more diffused bands were obtained with proteoglycan due to high content of sugars.

AGPs are reported to be present at different sub-cellular locations and in particular cell types and tissues. AGPs are found to be on outer surface of the plasma

membrane, bound to the cell wall or as soluble molecules in periplasmic space and plant secretions. Each of these is partially distinct and has partially overlapping structures. The localization at different location has functional significance. Therefore, in order to assign the possible role of AGPs it is important to know their localization inside cell. Immunogold labeling study using electron microscopy has been the method of choice to study the localization of AGP and used for reliable distinction between labeling in the cell wall and plasma membrane. Immunogold labeling study using electron microscopy showed that 46 kDa AGP isolated in the present work is a plasma membrane bound AGP. Membrane bound AGP has been reported from several plants and their function has been studied. Membrane bound AGP has been reported to play important role in cell signaling, pollen tube germination, and somatic embryogenesis induction. The possible function of the 46 kDa AGP obtained in the present work need to be explored.

**Expression of purified AGP under salt and other abiotic stresses**

The expression of AGP has been reported to be dependent on developmental stage and also affected by biotic and abiotic stresses. The expression of 46 kDa AGP under salt and other stress was studied using anti-AGP polyclonal antibody raised against 46 kDa AGP. Gradual decrease was reported in the expression level of 46 kDa AGP with increasing NaCl concentration in comparison to control. The expression of 46 kDa AGP was also repressed compared to control by various other abiotic stresses like KCl, mannitol, PEG and also in presence of ABA. The effect of NaCl stress on the expression level of 46 kDa AGP was also studied in ten different cultivars of groundnut. Differences were reported in the level of expression among various cultivars both in control and salt treated seedlings. However, the expression was repressed in salt treated seedlings of each

cultivars compared to control. The expression of 46 kDa AGP was also studied in root and shoot of control and salt treated seedlings. The expression was found to be reduced in both shoot and root under salt stress compared to control.

It has been reported from the salt adapted and un-adapted cell line of tobacco and other studies that reduced level of AGP expression help in salt stress adaptation. This is mainly by reducing cell wall extensibility by various possible mechanisms such as influence on the biophysical interaction between the plasma membrane and cell wall, or ferulic acid mediated cell wall polymer cross linking. In order to see whether there is any link between level of AGP expression and stress tolerance, the expression of AGP in control and salt treated seedling of draught tolerant and sensitive cultivars were analyzed carefully. It was revealed from the expression profile of AGP of control and salt treated seedling of different cultivars in general and the drought tolerant cultivar ICGS-37 in particular that AGP expression is associated with stress tolerance. The significantly low level of expression of AGP under salt and other abiotic stresses compared to control among different cultivars and the observation that the AGP expression was more reduced in salt treated seedling of drought tolerant line ICGS-37 compared to expression in its control seedling, indicated that this may be helping in its drought adaptation. Since the salt and drought responses are very similar it might also be helpful in salt adaptation. But in the present study no direct study has been performed to validate this point.

**Role of purified AGP in somatic embryogenesis induction**

AGPs from various sources have been reported to have somatic embryogenesis inducing potential and being used exogenously for study of somatic embryogenesis in different plants. The somatic embryogenesis inducing potential of 46 kDa AGP was

studied. Similar to the earlier observations, the exogenous addition of 46 kDa AGP to culture medium showed growth promoting and somatic embryogenesis inducing potential. It caused both the early induction of somatic embryos compared to hormone alone and also increases the number of embryos per explants. This is the first report of growth and somatic embryogenesis inducing potential of AGP from groundnut.

**Evaluation of antimicrobial activity of purified AGP**

A number of plant defense proteins have been identified. The best-known proteins involved in plant defense are lectins, pathogenesis-related proteins, hydroxyproline-rich glycoproteins, cyclophilin-like proteins, ribosome-inactivating proteins and protease inhibitors. The AGPs belong to class of hydroxyproline rich glycoproteins of plant origin and their immunomodulatory potential is well established and there are several reports that immunomodulatroy activity of a number of medicinal plants preparation reported to be due to their AGPs constituents. Recently, antimicrobial activity of AGP from a few medicinal preparations has been reported. The antimicrobial activity of 46 kDa AGP purified from groundnut seedling was evaluated. Both crude fraction and 46 kDa purified AGP were found to have broad spectrum antimicrobial activity. The crude extract showed both the antibacterial and antifungal activity but the purified AGP had only antibacterial activity but no antifungal activity. The antifungal activity shown by crude protein fraction could be due to other non-AGP present there in, as there were several proteins in crude preparation revealed by SDS-PAGE. These proteins were removed during AGP purification and resulted in loss of antifungal activity. The antibacterial activity was found to be broad spectrum as it was observed against all the bacteria tested namely *Serratia marcescens*, *Pseudomonas putida*, *Staphylococcus aureus* and *Bacillus*

*subtilis*. The MIC of the crude protein and purified AGP was found to be in the range of (3-4 µg/ml) and (6-8 µg/ml), respectively. This was very significant compared to the MIC (20-100 µg/ml) of other antimicrobial proteins reported earlier. The cell surface morphology study using SEM of the AGP treated and untreated bacteria, showed that AGP has great influence on morphology of the bacterial surface. The untreated bacterial surface was smooth while the treated cell surface was rough and different kind of deformity could be easily seen. At higher concentrations the cells were fully damaged and cell aggregates or debris could be seen. These observations were similar to the other earlier studies, and the mode of action is probably by membrane disruption as suggested in earlier studies. In conclusion, this study also indicates that 46 kDa AGP has antimicrobial activity and it is more likely causes some modulation at bacterial cell-surface that leads to cell lysis. This substantiates the earlier observation that AGPs have antimicrobial potential. This is the first report of isolation of AGP from groundnut and showing its broad spectrum antimicrobial activity. However, its actual mode of action still needs further investigation.

**Evaluation of AGP role as antifungal drug formulation**

Both the intact AGP and its purified polysaccharide fraction arabinogalactan (AG), are found to be highly water-soluble, possess a high degree of biocompatibility and used in a number of pharmaceutical and neutraceutical preparations. In one of the earlier study, a water-soluble injectable conjugate of amphoptericin B–arabinogalactan (AmB–AG) was formed using commercial preparation of AmB and AG. The conjugate was reported to be stable and found to increase solubility and stability of AmB in aqueous solution, significantly reduces its toxicity and possess a high degree of biocompatibility.

In the present study, the attempts were made to conjugate AGP with AmB in order to reduce its toxicity and to enhance its efficacy if possible. The AGP-AmB conjugate was prepared by a simple two steps schiff base reaction and its antifungal activity and cyto-toxicity were tested. The conjugate was found to be less toxic compared to AmB and there was no reduction in its antifungal potential. Therefore, AGP was found to be suitable carrier for AmB formulation without affecting its antifungal potential but increasing its water solubility and thus reducing its toxicity.

**Conclusions:** In the present study, a 46 kDa AGP from groundnut seedling was purified. Its AGP nature was confirmed by its reaction with Yariv reagent and also with AGP specific monoclonal antibody LM-2. Its expression was greatly reduced under salt and other abiotic stresses. It was found to be associated with salt stress adaptation. It has growth promoting and somatic embryogenesis inducing potential. This is the first report of AGP purification from groundnut and providing evidence for its role in growth promotion and somatic embryogenesis induction. Besides, it has significant broad spectrum antibacterial activity. This is also the first report showing antimicrobial activity of AGP from groundnut. This also acts as good carrier molecules in antifungal drug formulation by conjugation with AmB. The conjugate was found to reduce the toxicity of the drug by enhancing its water solubility without affecting its antifungal activity.

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