ROLE OF ACETYLCHOLINE IN SIGNAL TRANSDUCTION IN PLANTS

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Certificate

This is to certify that the thesis entitled **ROLE OF ACETYLCHOLINE IN SIGNAL TRANSDUCTION IN PLANTS** submitted in partial fulfillment of the requriement for the degree of Master of Science (Agriculture), in Crop Physiology to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by **Miss. Geetha Govind** under my guidance and supervission and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bangalore
September 2000

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INTRODUCTION

Chapter I

INTRODUCTION

In recent years there has been a spurt of activity relating to the use of plants for medicinal and other pharmaceutical purposes world-over. There has been an upsurge of research into the chemicals plants contain and the possible functions rendered by them in plants. For example, a large number of plants are recommended to be used as laxatives almost routinely by indigenous health care systems. But the evolutionary significance of such chemicals was not known until very recently when it was demonstrated that laxatives in plants might have evolved to aid the safe dispersal of seeds (Uma Shaanker et al.,1997). These studies have formed exciting platforms to address similar questions regarding a score of other chemicals contained in plants.

One such, and perhaps an extreme one, concerns the discovery of neurotransmitters in plants. Neurotransmitters are chemicals that help transmit signals among nerves or from nerves to muscle tissues in animal systems. In fact they form the core of stimuli-response reactions in animal systems. Briefly, an external stimulus when sensed sends forth a signal that is transmitted through neurons, which in turn generate action potential. The action potential on reaching the end of the neurons releases chemical substances known as neurotransmitters. The neurotransmitters then help in the transmission of the signals at junctions between nerves and between nerves, muscles and organs.

Until 1970s, neurotransmitters were thought to be in the animal domain only, which with their well-developed nervous systems were suitable candidates for neurotransmission. However, since the discovery of acetylcholine, one of the major neurotransmitters, there has been a world-view change in our perspective of neurotransmitters and transmission of signals in plants. Obviously nervous system is absent in plant. Nevertheless, plants are known to exhibit extremely rapid movements such as that exhibited in the Venus fly trap and *Mimosa pudica.* These plants can propagate electrical stimulus as efficiently as lower animals that have well-developed nervous systems. Plants are known to actively indulge in searching and foraging behaviour just as in animals. The movement of tendrils in certain species of plants, the searching of parasitic stems for host in a number of plant species and opening and closing of leaf and leaflets provide a suite of examples in which the role of signal transducers are involved. In all these situations, the speed of the respective movements might range from few seconds to few hours. It has long been known that carnivores plants and plants which are able to execute rapid movements can be stimulated locally and that such stimuli may be propagated from one organ to the other. This propagation is accompanied by a characteristic action potential wave similar to that which occurs when a nerve is stimulated. In other words, it would be interesting to enquire if the emergence and propagation of this action potential should follow the same rule in plants as in nerves of animals.

Nerve cells transmit electrical impulses or signals by opening pores in their membrane. In response to various stimuli, certain specific receptors on membrane undergo conformational change, which open ion channels and consequently change the electric polarity of membrane. This localised change in polarity of membranes thus induced in the vicinity of the receptor leads to a quick transient potential change and is termed as action potential. However action potential is fired only if the magnitude of signal received by the cell crosses a particular threshold. Therefore action potential is an " all- or- none" type of response.

The action potential in nerve cells is a depolarization process based on the prompt opening of Na^+ channels, leading to a large influx of Na^+ ions driven by electrochemical gradient. The process continues in a self-amplifying manner until the membrane potential has shifted from a negative resting value to positive Na^+ equilibrium potential. The recovery process is hastened by voltage gated K^+ channels. In plants the change in membrane potential is brought about in a similar manner. Except that instead of Na^+ and K^+ it is the diffusion of Cl^- and K^+ which decide the membrane excitability. In many cases Ca^{2+} is also known to be involved.

The basic molecular processes during action potential in plants however are poorly understood. The morphological structure of plant cell consisting of large vacuole, tonoplast, plasmalemma and cell wall doesn't facilitate measurement of membrane potential. The stimulus for firing an action potential could be physical, chemical or electrical. During transmission the nature of stimulus may change from one form to other for example, physical to chemical or chemical to electrical and vice versa. Therefore the language of message depends on frequency of action potential. Neurotransmission in animals is brought about by chemical substances such as acetylcholine and biogenic monoamines (e.g. Dopamine, dopa, noradrenaline, seretonin etc.), most of which have also been reported in plants.

Among the various neurotransmitters, the action of acetylcholine (ACh) is fairly well understood in animals. The ACh is reported to be present in more than 50 plant species belonging to major systematic groups. It has been detected in various plant parts, in aerial parts of angiosperms i.e. stems, hypocotyl, leaves, flowers and seeds. It is also detected in lower organisms like bacterium and fungi. There are reports on the presence of acetylcholine, its receptors and enzymes involved in its synthesis and degradation in plants. Recently receptors for two other neurotransmitters, glutamate and GABA (gamma- amino- butyric acid) have been characterised in plants; these have a high degree of homology with receptors found in animals (Gloria Coruzzi et al., 1998).

A number of workers have attempted to elucidate the role of the various neurotransmitters in plants. For example acetylcholine has been reported to be involved in a number of signaling processes in plants, primary among them being in the phytochrome mediated action. Besides, ACh has also been reported in processes such as change in electrical potential of root tips, leaf movements, membrane permeability to ions, flowering, seed germination, blue light effects in plants and in certain plant growth features. However, the exact mechanism of action of ACh in the various processes is far from clear.

In this study, an attempt has been made to review the role of neurotransmitters in plants with the specific objectives of:

a) **Elucidating the basic patterns associated with the accumulation and occurrence of neurotransmitters in plants:**
   Information on occurrence of ACh in different parts of various plant species belonging to different families and the medicinal use of the respective plant and physiological action of ACh and other neurotransmitters were collected. Based on the existing information an attempt has been made to understand the evolutionary significance of neurotransmitters in plants.

b) **To study the role of neurotransmitters in a few physiological processes and the mechanism underlying the involvement of ACh:**
   In this objective, the role of ACh in pollen grain germination and tube growth and stomatal regulation is studied.

c) **To study the role of neurotransmitters in tactile motion in plants:**
   The role of neurotransmitters in the coiling behavior of tendrils of cucurbits and the host acceptance or rejection by the parasitic stems of Cuscutta were also investigated.

REVIEW OF LITERATURE

Chapter II

REVIEW OF LITERATURE

Literature on acetylcholine and other neurotransmitter primarily relates to their role, distribution, metabolism and its evolutionary significance in plants. There are few studies on role of neurotransmitters in electrical signaling in plants. In this chapter an attempt has been made to review, the existing literature regarding acetylcholine, its occurrence in different parts of the plant, its distribution across genera and families and, it's possible role in signaling or any other physiological processes in plants.

There are numerous reports that show propagation of stimulus – changing or altering membrane potentials in plants much as that occurring in animal systems (Brinckmann and Luttage, 1974; Spanswick, 1972; Zawadzki and Trebacz, 1982; 1985). The electrical potential distribution has been measured precisely around the root surface of bean (*Vigna mungo*) (Yumi Watanabe *et al.*, 1995).

A stimulus on a plant may evoke excitation within the area of stimulus action and spread to the entire plant in the form of a potential wave. The electrical responses depend on kind of stimulus the plant or plant organ is stimulated with. However, electrical activity might also be generated spontaneously (Sibaoka, 1966; 1969; Mamulashvilli, Krasavina and Lialin, 1972; Pickard, 1973; 1974; Van Sambeck and Pickard, 1976).

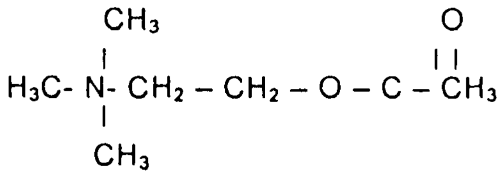
In animals external stimulus is sensed and signals are transmitted through neurons which generate action potential. The language of the message being transmitted depends on the frequency of action potential (Stevens, 1979). Action potential on reaching the end of the neurons releases chemical substances known as neurotransmitters. Acetylcholine is one of the important neurochemical involved in transmission of signals at junctions between nerves and between nerves and muscles (Nachmansohn, 1976). A truly rapid communication system, like that found in nerves, is obviously not present in plants, however cells of plants that exhibit rapid movements such as *Dionea muscipula* (Venus fly trap) and *Mimosa pudica* (touch-me-not) can propagate electrical stimulus as efficiently as nerves of lower animals (Sibaoka, 1969). Such rapid movements are mediated by action potentials (Sibaoka, 1969). There are reports on transmission of electrical signals or fluctuations in non-seismonastic plants such as *Cucurbita pepo, Cucurbita maxima, Phaseolus multiflorus, Helianthus annuus, Heracleum sibricum, Naravelia zeylanica* and *Luffa cylindrica* (Pickard, 1974; Takashi and Masashi Tazawa, 1989). The physiological role of electrical signals in such communication processes however is still obscure.

Work on animals systems has shown that bioelectric potentials are associated with ion fluxes, which in turn are often induced by neurohumor (neurotransmitter) namely, acetylcholine, norepinephrine, serotonin, dopamine, epinephrine, oxytocin, vasoprenin, glutamate, GABA (gamma amino butyric acid), tyrosine, L- DOPA, octopamine, aspartate, glycine, alanine, proline, cholecystokinin, neurotensin, enkephalins, somatoshetine and histamine (Goodman and Gillman,). The development and propagation of bioelectric potentials similar to the ones in nervous system of animals have been known for several decades in plants like Nitella and *Mimosa pudica*.

Acetylcholine

Acetylcholine (ACh) belongs to the cholinergic system of neurotransmitters and occurs at synaptic and neuromuscular junctions (Lester, 1977). It is responsible for accurate transmission of the action potentials between the pre- and post-synaptic membranes through synaptic cleft (Dunat and Israel, 1985; Lester, 1977).

Structure:



$$H_3C-\overset{CH_3}{\underset{CH_3}{N}}-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$$

The cholinergic system is composed of the following components:

1. Acetylcholine (ACh), an ester of acetic acid and choline, is released from the presynaptic membrane when an action potential reaches the end of the neuron and depolarises the membrane. Depolarization of membrane results in opening of calcium channels, calcium enter into the nerve and triggers the exocytosis of ACh containg vesicles (Lester, 1977).

2. Cholineacetyltransferase (ChAT), an enzyme catalysing the synthesis of ACh from acetyl coenzyme A and choline (Israel and Manaranche, 1985).

3. Acetylcholine receptors (AchRs) are localized in the postsynaptic membrane in animal cells. There are two types of AChRs, so called 'nicotinic' (nAChR) and 'muscarine' (mAChR) receptors (Changeux *et al.*, 1984; Dunat and Israel, 1985; Rotter, 1984; Stround and Finer-Moore, 1985). Both of these are stimulated by ACh and also by nicotine and muscarine respectively. ACh released from the pre- synaptic neuron bunds to the ACh receptors located on post- synaptic membrane and result in opening of various ion channels and inturn result in depolarisation of muscle membrane. AChRs activity can be inhibited by ACh antagonists such as atropine and D-tubocuranine (Sastry and Sadavongvivad, 1979).

4. Acetylcholinesterase (AChE), an enzyme occurring in the pre and post-synaptic membranes, responsible for hydrolysis of ACh to choline and acetic acid residues. ACh is relased from the receptor by the activity of AChE (Dunat and Israel, 1985; Israel and Manaranche, 1985).

Acetylcholine is synthesized in the nerve endings and stored in synaptic vesicles. Such vesicle contains about 10,000 molecules of ACh (Dunat and Israel, 1985; Israel and Manaranche, 1985; Changeaux *et al.*, 1984). The ACh-AChRs complexes, through their effect on the permeability of membranes to ions, initiate the development of action potentials on the post-synaptic membrane (Lester, 1977).

Further ACh is removed from AChRs with the participation of AChE and the choline and acetic acid resulting from its hydrolysis is reabsorbed from synaptic cleft into the synaptic endings (Dunat and Israel, 1985; Israel and Manaranche, 1985).

Discovery of Acetylcholine in Plants

In 1914, Ewins discovered acetylcholine in non-animal cells. He isolated it from ergot, a fungus. Several pharmacologists in 1920's and 30's reported the presence of ACh in both fresh and fermenting plant juices and in bacteria (Guggenheim, 1958; Whittaker, 1963). Since then it has been detected in many species of lower and higher plants as well as in bacteria and fungi (Fluck and Jaffe, 1976; Hartmann and Gupta, 1989).

However, Oury and Bacq (1937) could not detect ACh in 36 species of fungi, including ergot and attributed the reported presence of ACh in plants to bacterial contamination. Due to awareness that bacteria could produce ACh, a number of other reports on the presence of ACh in plants either could not be confirmed (Whittaker, 1963). The highest concentration of ACh has been found in some nettle species (Emmellin and Feldberg, 1949; Saxena *et al.*, 1965; 1966). In the 1970's ACh was detected in mung bean (Jaffe, 1970) and *Albizzia julibrissin* (Satter *et al.*, 1972) by the calm heart bioassay and paper chromatography and in most callus by employing both the frog heart bioassay and paper chromatography (Hartmann, 1971).

Hartmann and Kilbinger (1974a) confirmed the presence of ACh in moss callus by using gas liquid chromatography. ACh was detected in *Amaranthus caudatus, Cucurbita pepo, Helianthus annuus, Phaseolus vulgaris, Pisum sativum, Sinapis alba* and *Spinacia oleraceae* (Hartmann and Kilbinger, 1974b).

Gupta (1983) summarised the reports on presence and absence of ACh in plants. The list of ACh containing plants consists of three species of fungi, one species of bryophyte, one species of gymnosperm and 36 angiosperms, in addition to two species of bacteria and fermenting juices of many plants

Distribution of acetylcholine in the plant kingdom

Acetylcholine has been found in the tissues of more than 50 plant species belonging to all major systematic groups. Acetylcholine was detected in fungal hyphae (Ewins, 1914), in callus cultures of bryophytes (Hartmann, 1971; Hartmann and Kilbiger, 1974a) and in aerial parts (Hartmann and Kilbinger, 1974b; Jaffe, 1970; Miura and Shih, 1984), tubers and roots of higher plants (Emmelin and Feldberg, 1947; 1949; Hartmann and Kilbinger, 1947b; Jaffe, 1970; Miura and Shih, 1984).

In aerial parts of angisoperms, it has been found in stems (Emmelin and Feldberg, 1947; Hartmann and Kilbinger, 1974b; Jaffe, 1970; Miura and Shih, 1984), hypocotyls (Hoshino, 1983b; Jaffe, 1970), leaves (Hartmann and Kilbinger, 1974b; Jaffe, 1970; Miura and Shih, 1984), apical buds (Jaffe, 1970), flowers and seeds (Miura and Shih, 1984; Fiedler *et al.*, 1953).

Distribution of acetylcholine in different plant organ

Precise analysis of ACh distribution in plants has been carried out in four species; *Urtica dioica* L. and *U.urens* L. (Emmelin and Feldberg, 1949), *Artocarpus integra* Merr. (Lin, 1957) and *Phaseolus aureus* Roxb (Jaffe, 1970). In *Urtica urens* L. ACh had a similar concentration in all organs, while in *Urtica dioica* L., ACh levels differed in different plant organs. The highest ACh concentration was found in stinging hair, in the cortex and in phloem. The lowest concentration was found in the pith of the apical parts of nettle shoots (Emmelin and Feldberg, 1949).

In *Artocarpus integra* Merr (Lin, 1957) the highest ACh concentration was found in the youngest leaves. Within the leaf, a higher ACh level was recorded in the vascular tissue compared to that in the lamina. In the leafless shoot of *A.integra* the highest ACh concentration was observed in the pith and the lowest in the xylem. In all the *A.integra* organs studied there was a correlation between ACh level and the age of the organs. Lin (1952) suggested that an ACh concentration gradient exist in the aerial and underground parts of the plant. He demonstrated that in *Phaseolus aureus*, the highest ACh concentration occurs in the growing regions of roots and shoot. Hartmann and Kilbinger (1974b) have reported that, invariably, all plants contained more of ACh in the above ground than in the below ground parts.

Acetylcholine concentration in plants varies from fractions of nanomols (nanograms) (Miura and Shih, 1984) to hundreds of micromols or microgram (Jaffe, 1970; Lin, 1957) per gram fresh weight of the tissue. The plants in which highest ACh concentration has been found are different nettle species (Emmelin and Feldberg, 1947; Saxena *et al.*, 1965) and the cells with the highest ACh content are the stinging hairs (Saxena *et al.*, 1965). Large amounts of ACh are found in many medicinal plants such as *Viscum album* L., *Digitalis purpurea* L., *D.lanata* L. (Neuwald, 1952), *D.ferruginea* L. (Tulus *et al.*, 1961) and *Carum copticum* Benth (Devasankaraiah *et al.*, 1974).

Acetylcholine, norepinephrine and serotonin were known even prior to Jaffe's observation to be natural constituents of many plant extracts (Guggenheim, 1958). These biogenic amines were considered to be intermediate products of synthetic or degradative pathways in plants.

Distribution and localization of cholinesterase in plants

In nerves ACh is hydrolyzed to choline and acetic acid by the enzyme AChE (E.C. 3.1.1.7). However in non-neuronal locations hydrolysis of ACh takes place with the help of another enzyme pseudo cholinesterase (EC 3.1.1.8) but at a slower rate. Both the enzymes are inhibited by carbamates, such as eserine and neostigmine and by organophosphates such as diisopropyl fluorophosphate (DEP) and malathion.

The existence of acetylcholine in plants, provoked the search for enzymes responsible for the synthesis and hydrolysis of the enzyme. The enzyme that decomposes ACh has been found in Nitella cells (Detlbaen, 1962) and in representatives of Cruciferae, Solanaceae and Leguminosae. According to a list compiled by Gupta (1983), the absence of ACh hydrolysis has been recorded in 65 species of plants and the capacity of ACh hydrolyses has been reported in 103 species of green plants, two species of bacteria and one slime mold. Conclusive proof for the presence of cholinesterase based on kinetic properties and response to inhibitors are available in only six species of which four belong to the Leguminoseae – *Pseudomonas fluorescens* (Fitch, 1963a, 1963b; Laing *et al.*, 1967), *Phaseolus aureus* (Riov and Jaffe, 1973a; 1973b), *Solanum melongena* (Fluck and Jaffe, 1975), *Pisum sativum* (Kasturi and Vasantharajan, 1976), *Phaseolus vulgaris* (Mansfield *et al.*, 1978; Ernst and Hartmann, 1980) and *Cicer arietinum* (Gupta and Maheshwari, 1980). It was also found in etiolated maize and oat seedlings (Fluck and Jaffe, 1975; Tretyn and Tretyn, 1990).

A survey was conducted by Gupta and Gupta (1997) regarding ChE activity in plants by employing *in vivo* test in 106 species belonging to 53 families of Angiosperms, 7 species belonging to 5 families of Gymnosperms and 5 species belonging to 5 families of Pteridophytes. AChE was not found in all plants, and not all parts showed ChE activity.

Cholinesterase activity was tested spectrophotometrically in the cell free extracts of ten marine algae belonging to chlorophyta, phaeophyta and Rhodophyta. All the algae showed ChE activity (Gupta *et al.*, 1998).

Rivov and Jaffe (1973a, 1973b) have found that phosphon-D, AMO 1618, CCC and 9-80 to be inhibitors of ChE activity. Of these AMO 1618 was found to be a potent, specific, non-competitive and irreversible inhibitor of this enzyme.

Localization of acetylcholinesterase in plants

AChE activity has been recorded in all parts of the seedlings in mung bean (Fluck and Jaffe, 1974a) and Bengal gram (Gupta, 1983). The AChE activity in shoots has been found to decrease with the growth of seedlings. Almost 90 per cent of the AChE activity in seedlings of *Cicer arietinum* is found in roots (Gupta, 1983). Cholinesterases were extracted from plant tissues using high ionic strength solutions of $(NH_4)_2SO_4$ (4%) or 6 x 10^{-1} M KCl (Riov and Jaffe, 1973a). The enzymes from *Solanum melongena* L. and *Zea mays* L.

can be extracted with a low ionic strength buffer, without $(NH_4)_2SO_4$ (Fluck as Jaffe, 1975). This may be evidence of structural differences of the enzyme, or of their different subcellular localization.

The products of the ChE enzymatic reactions appear in the cell walls and the space between the cell wall and plasma membrane. Cell fractionation and electron microscopic studies in mung bean roots indicated that 95 per cent of the AChE activity was associated with the cell wall (Fluck and Jaffe, 1974a). Maheshwari *et al.*(1982) observed AChE activity in the cell wall and nuclei of the root cells of *Pisum sativum*.

Lee and Thompson (1975) reported that concentration of AChE in plasma lemma and cell wall increases with growth of seedlings of *Phaseolus vulgaris*. Hartmann and co-workers extensively studied the possible localization of AChE in the plasmalemma of bean tissue. They reported no AChE activity in protoplasts from beans, but the activity was exclusively found in the cell wall residue. Bean hypocotyl tissue contained 94 per cent of the enzyme activity in the cell fraction, but there was no activity in the cytosolic fraction. The same pattern was found for bean callus. Roshchina (1986) has also reported presence of AChE activity in the pea chloroplasts. Tretyn and Tretyn (1990) have described localization of AChE activity in etiolated oat Coleoptiles. In wheat aleurone cells the product of the enzymatic reaction was observed only on the external side of the plasma membrane (Tretyn *et al.*, 1986).

The assumptions that part of the plasmalemma and not the cell wall were the main site of AChE localization was not supported by studies with protoplasts. Preliminary immunological studies suggested the localization of AChE on the surface of naked bean protoplasts (Hartmann *et al.*, 1980; 1981) to be due to a non-specific reaction of the polyclonal antibodies against AChE, which also contained antibodies against Cholineacetyltransferase (Hartmann, Unpublished). No AChE activity was found in plasma membrane of bean protoplasts (Hartmann and Gupta, 1989). Tretyn and Kendrick (unpublished) also found no activity of this enzyme on the plasma membrane surface of etiolated wheat protoplasts, the activity being localized inside the proplastid

(Tretyn and Kendrick unpublished). However AChE was reported in pea chloroplasts by Roshchina and Mukhin (1985) and Roshchina (1988). Recently Bednarska and Tretyn (1989 reported AChE activity on the surface of stigmas of *Pharbitis nil* (L.).

Comparison of plant (Acetyl) cholinesterase and animal acetyl cholinesterase

There are a number of molecular and biochemical properties which are similar between plant ChEs and animal AChEs. The molecular mass of plant ChEs is higher than 200,000 or 800,000 Dalton or lower than 80,000. These values are comparable to the molecular masses of polymers and protomers of animal AChEs (Ott, 1985). Another common feature of these enzymes is their tendency of aggregation *in vitro*. Plant ChEs have a high affinity for ACh and therefore their preference for hydrolyzing it when compared to other choline esters. The membrane localization of plant ChEs as well as their molecular and biochemical properties indicates that these enzymes are AChEs. The fact that plant and animal ChEs are so similar suggests they perform the same function in regulating the level of ACh in plants and animals.

Synthesis of Acetylcholine

Universal presence of ACh in plant tissues indicates that the neurotransmitter might be synthesised in situ in plants. In animals ACh is synthesised from acetyl – coenzyme A (acetyl – CoA) and choline with the participation of ChAT (Choline acetyl transferase) (EC 2.3.1.6) (Dunat and Israel, 1985). Choline as well as acetyl-CoA has also been detected in plants (Miura and Shih, 1984; Tretyn and Tretyn, 1988).

The activities of choline kinase (EC 2.7.1.32), phosphorylcholine – Cytidyl transferase (EC 2.7.7.15) and phosphoryl choline diglyceride transferase (E.C. 2.7.8.2) was found in extracts of etiolated bean seedlings (Hock and Hartmann, 1981). These enzymes catalyze transformation of choline to phosphatidyl choline, one of major components of phospholipids in biological membranes (Hock and Hartmann, 1981).

Occurrence and localization of choline acetyl transferase (ChAT) in plants

Preliminary attempts to detect ChAT in plants failed (Riov and Jaffe, 1972a). First report of ChAT activity in plants came from Barlow and Dixon (1973) who used buds and young leaf hairs of stinging Nettle (*Urtica dioica*) which are extremely rich in ACh (Emmelin and Feldberg, 1949), but they were devoid of AChE activity (Emmelin and Fedlberg, 1947). The activity of the enzyme was determined by measuring the rate of ACh synthesized from choline and acetyl CoA. The concentration of ACh developed was determined by bioassay (Barlow and Dixon, 1973), and by using radiotracer method (Smallman and Maneckjee, 1981).

Choline acetyl transferase presence was also claimed in extracts of pea buds, cauliflower and bean hypocotyl hooks (Biro, 1978). It was also found in spinach (*Spinacia oleracea* L.), sunflower (*Helianthus annuus* L.) and a blue green alga (*Oscillatoria agardhii*) (Smallman and Maneckjee, 1981) as well as in seeds of plants belonging to the genus Alluim (Hadacova *et al.*, 1981). The enzyme was isolated and further characterized from bean seedlings (*Phaseolus vulgaris*) by Hoffmann and Hock (1983). Maximum ChAT activity was found at 40°C (Barlow and Dixon, 1973; Smallman and Maneckjee, 1981) and around pH 9.0 (Barlow and Dixon, 1973). The highest enzyme activity was exhibited in the extracts from the youngest parts of nettle i.e., from buds and young leaves (Barlow and Dixon, 1973; Smallman and Maneckjee, 1981). The specific activity of the enzyme isolated from young nettle leaves was similar to that exhibited by ChAT isolated from neural tissues of insects (Smallman and Maneckjee, 1981). The specific activity of the enzyme isolated from plants other than nettle was about 100 times lower than that of ChAT obtained from nettle tissues (Smallman and Maneckjee, 1981).

Acetylcholine receptors

No direct evidence has yet been obtained to confirm the presence of AChRs in plants. AChRs have not been isolated from plants or any other non-nervous system. Fluck and Jaffe (1970) assumed that plant AChRs might have two types of biochemical function. They may show the properties of a non-enzymatic regulatory protein controlling membrane permeability or they may be linked with enzymes controlling specific metabolic pathways. In preliminary studies on extracts from bean roots Fluck and Jaffe (1976) demonstrated the presence of ACh binding sites and a protein with strong affinity for ACh in the cell wall fraction of *Phaseolus vulgaris* (Hartmann *et al.*, 1981; Hartmann and Gupta, 1989). They also found AChE and ChAT activity in the extract but no work was carried out on biochemical characterization of the ACh binding sites (Jaffe and Hartmann *et al.*,1980).

The use of AChR antagonists (atropine, D-tubocuranine) and agonists (Muscarine, nicotine) of ACh has provided indirect evidence of the existence of AChRs in plants. Atropine increases the adenosine triphosphate (ATP) level in the bean buds (Kirshner *et al.*, 1975) and the activity of choline kinase (Hartmann and Schleicher, 1977). It neutralizes the stimulating effect of red light and ACh on the changes in growth pattern of *Vigna* seedlings (Hoshino, 1983a), counteracts the Ach stimulated shrinking of the cells of bean pericycle (Toriyama, 1978), nullifies the indole-3-acetic acid (IAA) stimulated flowering in *Lemna gibba* G_3 (Hoshino, 1979) and inhibits the incorporation of the ^{32}P into the phospholipids of bean hypocotyls (Hartmann *et al.*,1980). Atropine affects germination of photoblastic seeds and the synthesis of flavonoids in barley tissues (Saunders and McClure, 1973). On the other hand, it slightly reduces the GA stimulated elongation of cucumber hypocotyls (Verbeek and Vendeig, 1977) and has no effect on the auxin stimulated elongation of oat coleoptile sections (Evans, 1972).

Other neurotransmitters

Several other neurotransmitters namely norepinephrine, seretonin, glutamate, GABA and dopamine etc. are known to be actively involved in the transmission of signals in the animal system (Anelrod, 1974). Of these, a few have been reported in plants too. For example, quite recently, the receptors for the neurotransmitters, glutamate and GABA were found in plants Indeed, the genes for glutamate receptor were cloned in plants and their role in light signal transduction has been studied (Gloria Coruzzi *et al.*, 1998).

Physiological role of ACh

Acetylcholine has been shown to be involved in a number of physiological processes of plants. In many cases it mimics the effects of red light. It is also known to be involved as an messenger of phytochrome action. Some of the physiological processes in which role of Acetylcholine has been reported are : a) the processes associated with membrane permeability (Jaffe 1968, 1970), b) mimicking the effect of red light, c) in movement of leaves of *Mimosa pudica, Albizia julibrizin* and *Samanae saman* (Hartmann and Gupta, 1989; Satter et al., 1972), d) membrane permeability to ions like H^+ and other ions resulting in changes in bioelectrical potentials (Jaffe, 1970).

Few other processes which are reported to be affected by Acetylcholine are flowering in case of *Lemna gibba* (Kandela et al., 1972; Oota, 1977), seed germination of photoblastic seeds (Hadacova et al., 1981; Holm and Miller, 1972), pollen germination and tube growth (Chabbra and Malik, 1978), in plant growth (Kasturi, 1978; Dekhuijzen, 1973; Mukherjee, 1980), interaction with growth regulators such as IAA (Parpus, 1976) and GA (Dekhuijzen, 1973), mediation of ATP levels (Hartman et al., 1980), tendril coiling (Hua-BaoGuay et al., 1995), in stomatal movement (Madhavan et al., 1995) and also in heat stress signaling (Momonoki and Momonoki, 1993).

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The objectives envisaged in the study were accomplished by following literature survey and experiments were carried out at the laboratory of Dept. of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore. The study principally involved addressing the following questions:

a) To understanding the distribution profile of neurotransmitters in different parts of the plants and across plant species.

b) To examine the role of neurotransmitters in a few physiological processes and tactile motion in plants.

Accordingly, several different systems and methods were used to address the various objectives. These are briefly described below. All the chemicals required for the study were procured from Hi-Media Company.

Objective 1:

Basic patterns of occurrence of acetylcholine and the evolutionary significance of neurotransmitters in plants

a) Association between plants used in treatment of neural disorders and their levels of ACh

We examined the association between the plants used in the treatment of neural disorders and the occurrence of acetylcholine in them. Information regarding medicinal plants used in treatment of neural disorders was collected from published literature, Indian Materia Medica, and other archival sources. Information included the names of plants, their family, habit, plant part used, its action reported, presence of alkaloid, use in Ayurveda, Siddha and Unani etc.

The information so collected was structured in a relational database using Microsoft Access program and frequency distributions of the various data parameters were generated. The distribution pattern of plants across different habits and families were examined. Distribution patterns of the neurotransmitters in different plant parts was also examined. Finally, the physiological actions (viz. anaesthetics, anodynes, emetic, hypnotics, narcotic and nervine sedative) with respect to the different plant species used as curatives in neural disorders were analysed.

b) Pattern of accumulation of ACh and its enzyme systems in plants and their parts.

We examined the patterns of accumulation of ACh and its breakdown enzyme, Ach-esterase in plants and their parts. Information on the occurrence of ACh and ACh-enzymes were obtained from published papers, reviews and other archival materials. The data was analysed using a relational data base in Microsoft Access. The patterns were analysed with respect to the habit of the plant, family, genera and plant parts containing the neurotransmitter.

Objective 2: Role of neurotransmitters in physiological processes

To accomplish this objective, study was carried out on pollen grain germination and stomatal movement. Both of these processes are ion gradient dependent and thus mimic the ion gradients involved during the process of nerve impulses.

Sub-objective: Effect of neurotransmitters on pollen germination

We examined the role of neurotransmitters in sunflower pollen grain germination and tube growth. Well-developed protocol for the in vitro pollen grain germination and growth assay has been developed in the laboratory at

Department of Crop Physiology, UAS, Bangalore (Keshava Murthy, 1995). Further, because of the relatively photoinsensitive nature of the plant, the pollen grains for the experiment could be available irrespective of the seasons.

Sunflower plants (population : Morden) were raised in battery containers with recommended doses of fertilizer and irrigated at regular intervals. Once the plants started flowering the heads were covered with bags to get sufficient pollen grains and to prevent contamination by foreign pollen grains. Pollen grains were collected early morning between 8 – 8.30 a.m. using a brush and kept in cold condition until use to prevent the loss of viability. Before a pollen grain germination assay, the pollen grains were brought to room temperature and mildly thawed under an incandescant lamp.

The pollen grains were sprinkled into cavity slides containing 40μL of pollen germination media comprising of Calcium Nitrate (300 ppm), Boric acid (200 ppm), Magnesium sulphate (200 ppm), Potassium Nitrate (200 ppm) and Sucrose 15% along with extract of stylar tissue along with the respective treatments of neurotransmitters. An osmotic potential of –4 bars was maintained in the medium by using PEG 6000 to yield maximum germination of sunflower pollen grains. The cavity slides were placed in a petriplate and incubated for 30 minutes at room temperature; about 70 – 80% RH was maintained by lining the petriplates with moist filter paper.

The various nuerotransmitters used in the pollen grain bioassay were acetlycholine, adrenaline, glutamate, GABA and Dopa.

The role of ACh in pollen grain germination was confirmed using Atropine, an antagonist of the muscarine receptor site of Ach. The involvement of Ca^{2+} in Ach mediated pollen grain germination was studied using Ca^{2+} chelator (EGTA) and calcium transport blocker (Lanthanum chloride).

All treatments were either provided as pre-treatments or provided directly along with the other pollen grain germination medium. For pretreatment the pollen grains were incubated in either neurotransmitter or Atropine or Calcium chelator for 15 minutes then transferred to either pollen germination media or pollen germination media along with ACh and incubated at room temperature for 30 minutes.

Observations on per cent germination and tube length were recorded under 10x magnification so as to cover a large microscopic field. For each treatment, two replications were maintained and totally 15 fields for each treatment were recorded. Tube length was recorded using ocular micrometer, which was previously calibrated using a stage micrometer.

The mean and standard deviations of the various treatments were computed and analysed using a two-way ANOVA using MSTAT package.

Sub-objective: Effect of neurotransmitter (ACh) on stomatal movement

In this sub-section involvement of ACh on stomatal regulation was studied. The stomatal bioassay was performed with epidermal peels from fully expanded young leaves of *Commelina communis,* in which the stomata is relative large and peeling of epidermis is quite easy. The plants were raised in battery containers and the stomatal regulation studied by modifying the procedure of Trejo and Davies (1993).

1. Standardization of buffer for stomatal opening :

Experiments with stomatal system require a fairly standard bioassay system in which repeatable monitoring and evaluation of the effects of neurotransmitters could be made. For this an attempt was made to develop a buffer system in which there would be consistently high stomatal opening in the in the peels under natural light. The following buffers were tried : MES – KCl , Tris – KCl – $CaCl_2$ and KCl – $CaCl_2$ buffer.

The epidermis was carefully removed from abaxial surface of *C. communis* leaves and cut into strips of approximately 1 cm^2 area. The strips were incubated in different buffers adjusted to pH 6.15 for 30 minutes under dark, so that all the stomata are closed. From this homogenous pool, epidermal strips were picked at random and incubated in respective buffers under natural light (400 – 600 μ εin/m^2/sec) in petri plates. The petri plates were placed in glass cubicle measuring 2 x 1 x 2 ft. Carbon dioxide free air was pumped into the cubicle by passing air through 5N KOH or soda lime. Excess light which is photoinhibitory was cut off by allowing light to diffuse through a layer of water.

For each treatment two replications were maintained with 8 – 10 peels or strips per replication. Three strips were picked at random and all the stomata in the uniform fields were considered. Observations on stomatal aperture were recorded after 1 and 2 hr of incubation using ocular micrometers in the stereomicroscope and expressed in micrometers. For all experiments with neurotransmitters, 100 mM KCl + 0.1mM $CaCl_2$ buffer was used.

Effect of Ach on stomatal regulation (in vitro)

The protocol described above was followed with the epidermal strips of *Commelina* incubated in KCl – $CaCl_2$ buffer along with ACh at varying concentrations. After 1 and 2 hr of incubation, data was recorded on the per cent stomata open or close. Indirectly, evidence of the involvement of ACh was obtained by the use of Atropine, an antagonist of the ACh receptor. We also examined the dependence of ACh mediated action on calcium by using a Ca^{2+} chelator (EGTA) or a calcium channel blocker (Lanthanum chloride). The various treatments were either provided as direct treatments along with the stomatal buffer systems or were provided as pre-treatments; in the latter case the stomatal peels were incubated in the respective treatment solutions for 15 minutes and then incubated with ACh for 15 minutes and further transferred to the stomatal buffer system of KCl – $CaCl_2$.

Two replications were maintained with 8 – 10 strips for each of the treatments. Three strips were selected at random and three uniform fields were chosen for the observations. Observations on all stomata in that field were recorded using ocular micrometer. The per cent guard cells open and the extent of opening was recorded using an ocular micrometer. The data was treated statistically; mean and standard deviations of the various treatments were computed and a two-way ANOVA was performed.

Effect of ACh on stomatal regulation (at single leaf level)

The effect of neurotransmitter on stomatal regulation at single leaf level was examined by studying the rate of transpiration as influenced by the various neurotransmitters.

Young expanding sunflower leaves with their petioles were excised from sunflower plants and incubated in test tubes containing distilled water or neurotransmitter solutions for 3 to 6 hours. They were then transferred to water of known volume and at the end of 3 or 6 hr, the amount of water lost through transpiration was recorded. The experiment was conducted under natural light. Evaporational loss was minimised by plugging the test tubes with cotton, so that any loss in water, which occurs, can be attributed to transpiration loss alone.

Observations on water loss were recorded by calculating the difference between final and initial volume. Observations were recorded at every 1hr and continued for 6hr. The loss of water was calculated and expressed per unit leaf area. The mean and standard deviation in water loss was computed and the data was subjected to a two-way ANOVA.

Objective 3: Involvement of neurotransmitters in tactile motion in plants

In this objective, we examined the role of neurotransmitters in the tactile motion of plants. We examined the role of neurotransmitters in the tactile motion of tendrils in the Cucurbitaceae family and that of the stems of Cuscuta. Both of these systems, tendrils and stems of Cuscuta, are excellent example of tactile motion in plants. The motion in both of them would be under intense natural selection to either obtain support (in tendrils) or host (in cuscuta).

Effect of neurotransmitters on tendril coiling

We examined the effect of neurotransmitters on the coiling of tendrils in bottlegourd (*Cucurbitacae).* The material was procured from the plants growing in Botanical garden, GKVK, and Bangalore. The tendrils were excised using a sharp blade and floated in distilled water and brought to laboratory immediately before they coiled. They were cut into segments of 14 mm (from 5 – 19 mm region from the apical end) and incubated in test solution. This was done as rapidly as possible to avoid coiling of segments as coiling occurs within minutes. The test solution contained 0.75-mM K_2HPO_4 Citrate buffer, pH 5, 0.5 mM KCl, Chloramphenicol 50µg/mL (Rajgopal et al., 1988) with or with out the required neurotransmitter (Acetylcholine, GABA, Glutamate, Dopa) at varied concentration. The role of ACh was further confirmed using Atropine at varied concentration set arbitrarily based on ACh concentration.

The experiment was carried out in glass petriplates. For each treatment 2- 3 replication was maintained. Each replication contained 4 – 6 segments based on availability of tendrils. The plates were shaken simultaneously after each observation. The segments were classified based on the stage (or extent) of coiling they were in. The extent of coiling was measured in circular degrees (0^0 representing no coiling, < 180^0, 180^0, > 180^0, $360^{0,}$ > 360^0 (representing more than one complete coil).

Observations on number of segments in different stages of coiling were recorded at every 5 minutes interval until the coiling reached the last stage or coiling was maintained (0.45 – 1 hr). The percentage of segments or coils in different stages was calculated and the rate of coiling was determined.

Effect of neurotransmitters on coiling of Cuscuta

In this experiment, *Cuscutta reflexa* stems were cultured on *Tecoma stans* hedges in GKVK farm. For experimental purpose uncoiled free hanging vines with tip intact were used. 15 cm segments of free hanging vines were cut using sharp blade and brought to laboratory in distilled water and incubated in test tubes containing test solution as mentioned above along with different neurotransmitters.

Experiments were carried out using dead and live support to examine whether Cuscutta can sense the support and respond to it. For both the experiments (dead and live support) the Cuscutta segments were pretreated before tying to the support. For dead support an inert material (broomstick) was used, which was fixed to the testube. For live support stems of Tecoma (without leaves to avoid disturbance or error) were used; the live support was maintained in a test tube containing water to maintain its turgidity.

The Cuscutta stems after pretreatment were tied to the respective support with the help of a cotton thread to minimise damage. Observation regarding initial deviation or angle made by Cuscutta from support was recorded and further deflection or angle was recorded at every 15 minutes interval using a protractor. The observations were continued for 3 hr.

Change in angle made from the initial was calculated and percentage of stems or segments reaching or making an angle of 45^0 or 90^0 from the support was calculated. The results obtained were expressed as % acceptance or rejection. Movement of segments away from the support was regarded as rejection (change in angle is positive), while movement towards as acceptance (change in angle is negative).

RESULTS

Chapter IV

RESULTS

In this chapter the results of various objectives investigated in the study are presented. The first section presents the basic patterns of occurrence of acetylcholine or any other neurotransmitter in plants. An attempt has been made to explore the evolutionary significance of neurotransmitters in plants.

In the second section, results on the role of neurotransmitters in a few physiological processes is presented. In the final section the role of neurotransmitter in tactile motion in plants is presented. The term neurotransmitter is used in a generic sense to all the compounds that in some way enhance or alter the signal reception and the consequent action. Emphasis has been given to acetylcholine as a major neurotransmitter, while discussing the results of neurotransmitters.

Objective 1: Basic patterns of occurrence of acetylcholine in plants

Based on a relational database, information on the medicinal plants used in treatment of neural disorders was compiled with respect to their occurrence across habit, plant families, plant parts was examined. The physiological action reported in the species was also recorded.

Plants used in treatment of neural disorders

An inventory of the various neural disorders revealed that 16 different types of brain infection ranging from loss of memory to chronic neuritis exists. Plants have been used to cure most of these 16 neural disorders under different medical health systems. Among the most common neural disorder for which plants were used as curative were epilepsy and neuralgia. However the records of plants used under the ayurvedic system of medicine were very limited to specifically vatavyadhi, weakness and exhaustion. On the other hand records of use of plants for the treatment of convulsions, epilepsy, fainting, neuralgia, neuritis, excessive veney, neurathesmia were quite frequent under the Siddha and Unani system of medicine.

A total of 209 plant species are recorded to be used in curing neural disorder, but only 53 of them (25%) have records of being used traditionally in various medical systems. Of the 16 types of neural disorders that are recorded to be treated by plants, Ayurvedic medicinal system treats only two of them, while Siddha and Unani put together treat 10 neural disorders (Fig. 1 and 2). Based on the physiological action such as plant serving as anesthetic, narcotic etc. plants were classified as having one or the other function. Most plants were recorded to have an emetic function (110 species i.e. around 44%) followed by anodyne, narcotic and anesthetic. Only 2 species were reported to have an anesthetic effect as practiced in ayurvedic system of medicine. Over all, about 220 species of plants are reported to have one or the other physiological action. The physiological function rendered by plants in Ayurveda, Siddha and Unani system of medicine range from 1 to 6 (Fig. 3).

Association between plant families and curative properties

The association between the families represented by the plant and their curative properties was examined using the relational database. Prominent among the family that were associated with treatment of neural disorders were Cucurbitaceae, Liliaceae, Papilionaceae, Solanaceae, Valerianceae, Umbelliferae and Zingiberaceae (Fig. 4).

The enzyme AchE most frequently occurred in Asteraceae followed by Euphorbiaceae, Papilionaceae, Solanaceae, Moraceae and Apocynaceae (Fig. 5).

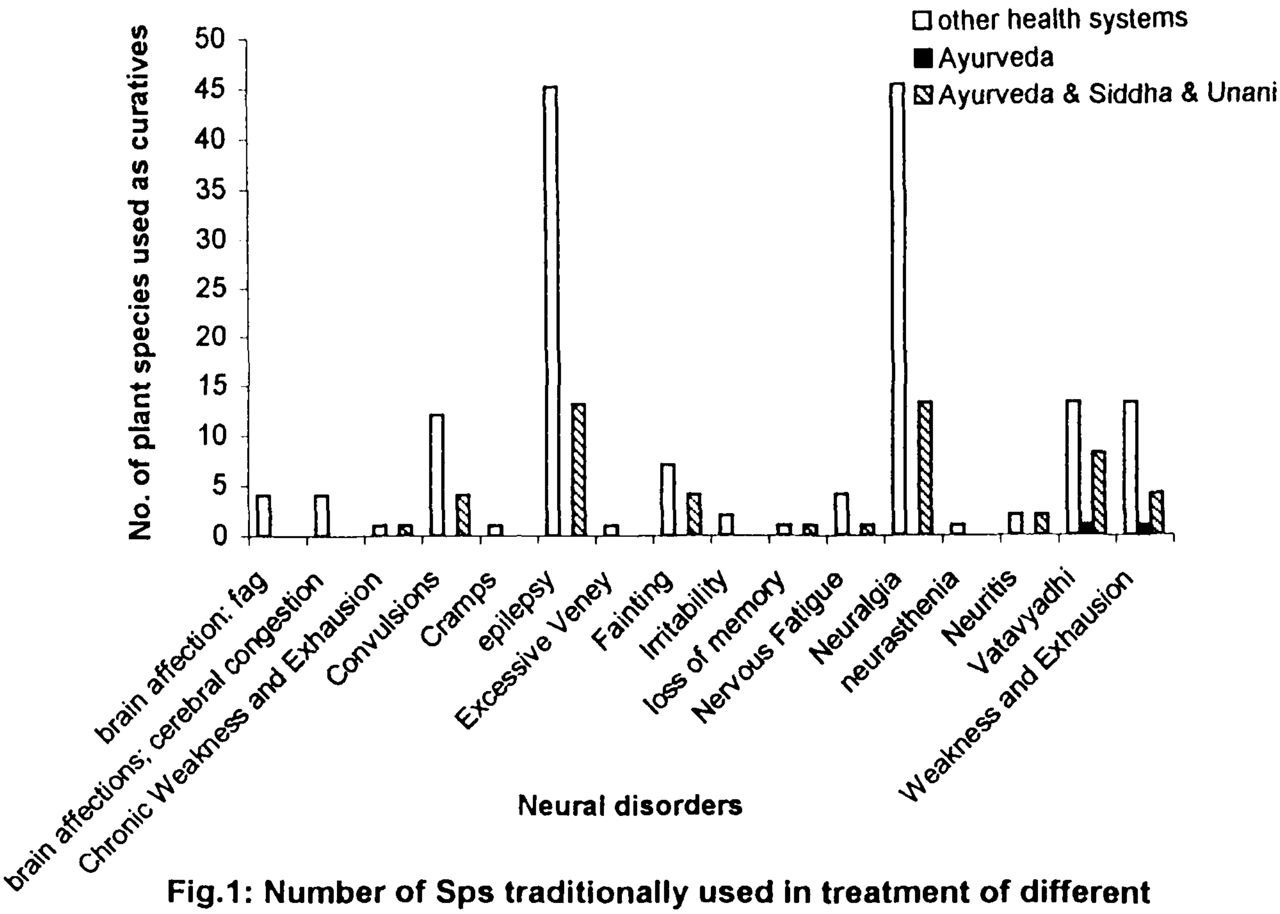


Fig.1: Number of Sps traditionally used in treatment of different Neural disorder.

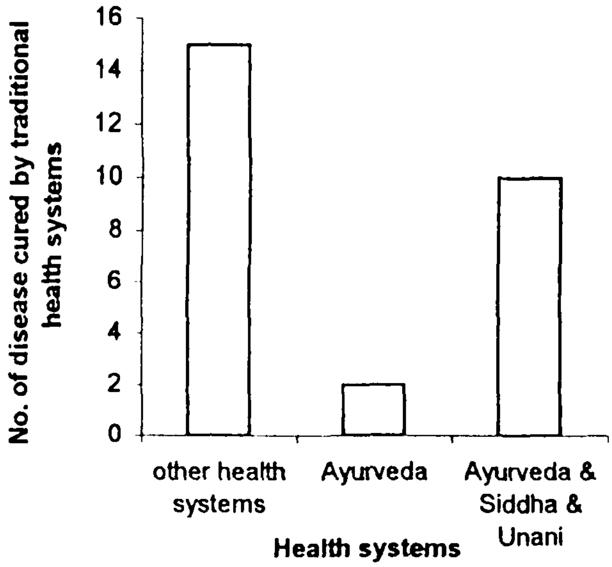


Fig. 2: Number of neural disorders treated by different traditional health systems. Others refer to those cited in Indian Materia Medica but not included in Ayurveda, Siddha and Unani health system.

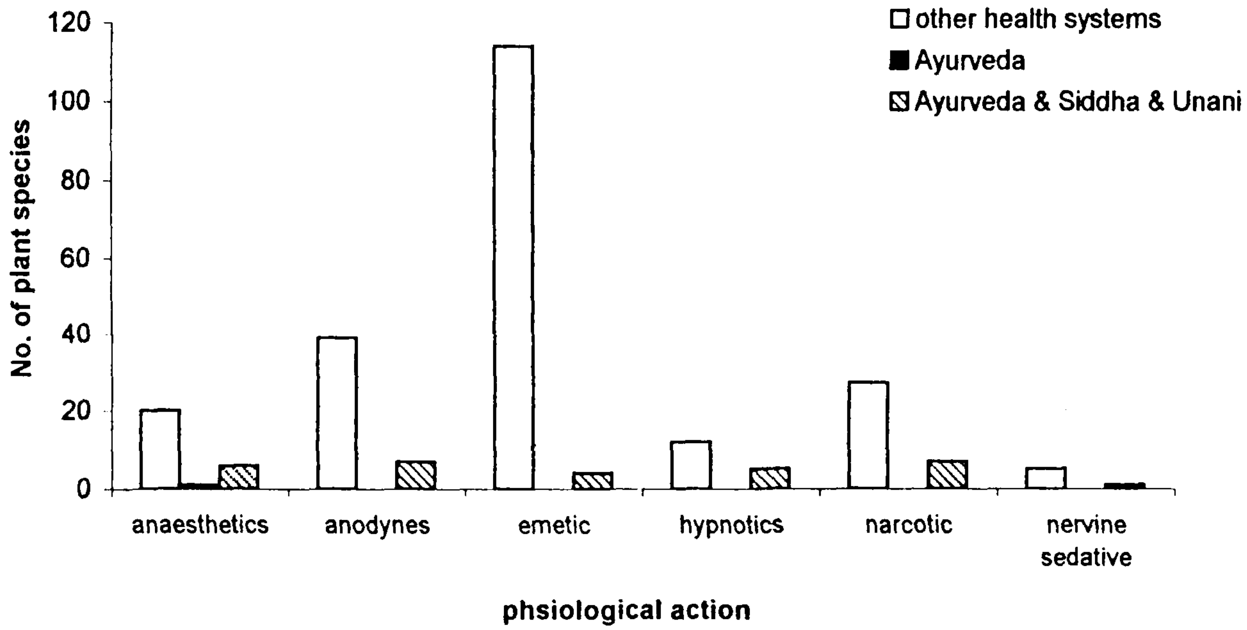


Fig. 3: Number of plant species used traditionaly for their physiological actions

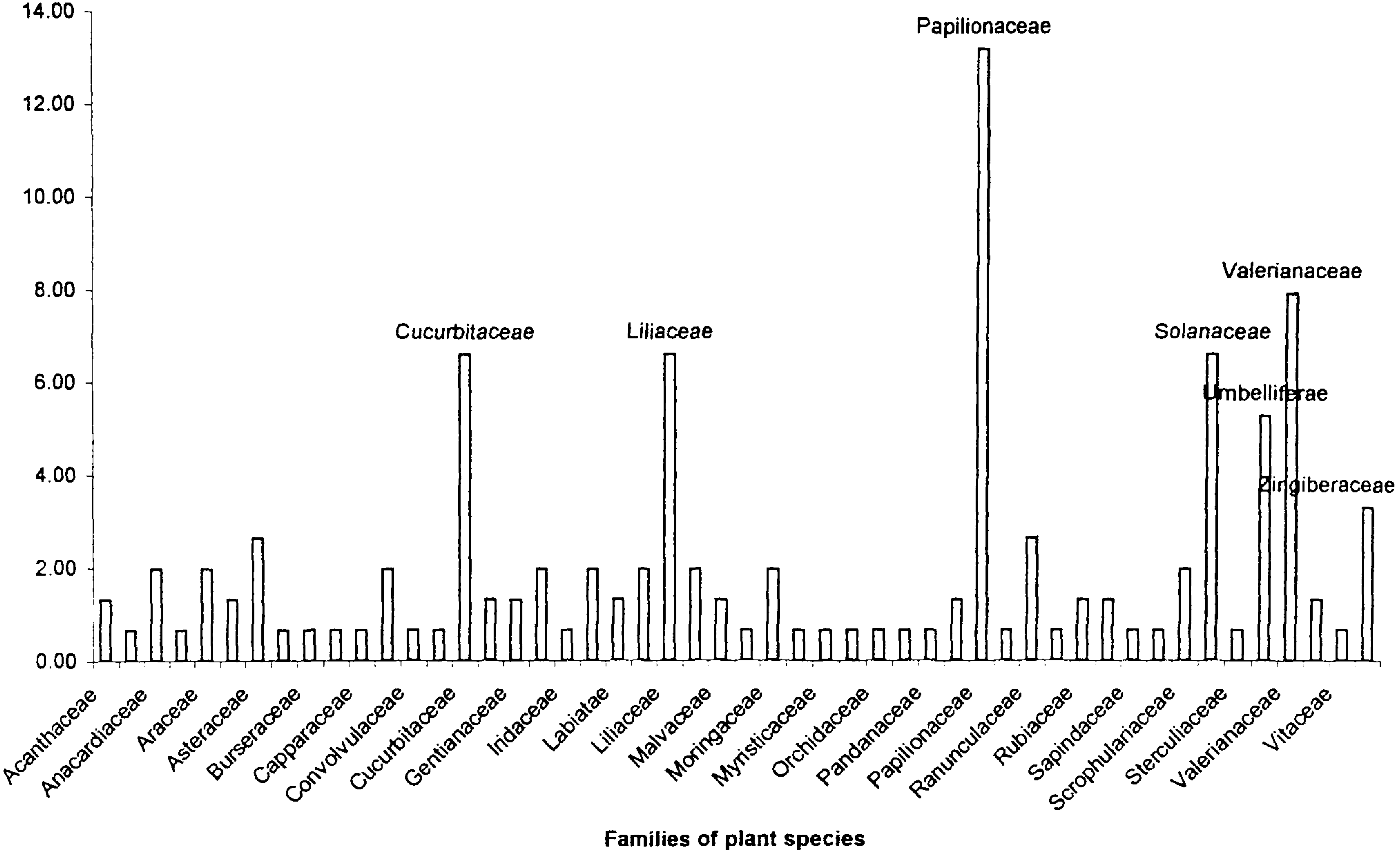


Fig 4: Percentage of families used in treatment of neural disorders.

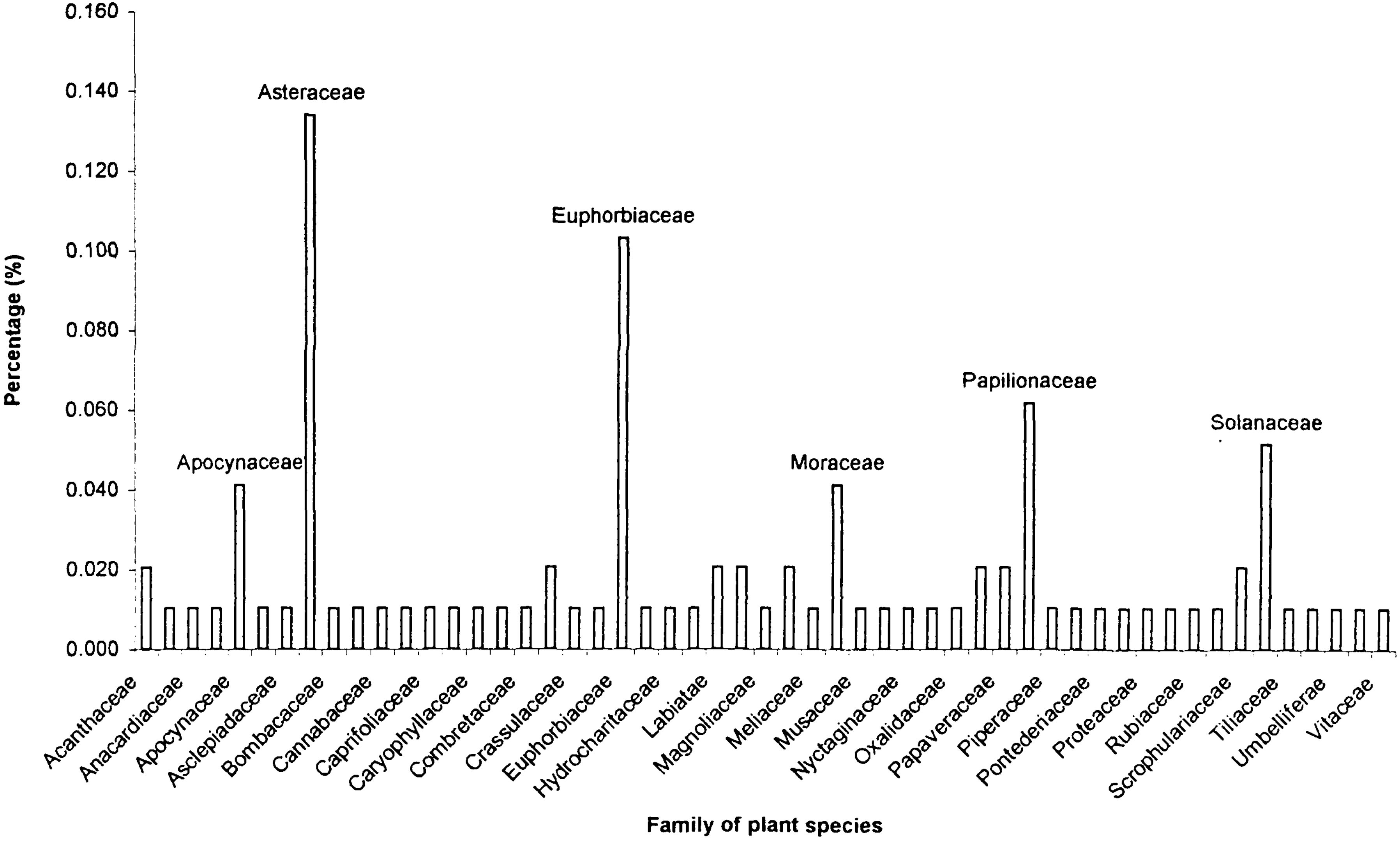


Fig. 5: Percentage of families in which acetylcholinesterase has been detected.

The presence of cholinesterase suggests an endogenous mechanism in plants to reduce the levels of the neurotransmitter, acetylcholine. A cursory examination of neural disorder and cholinesterase activity indicates that corresponding to a low frequency occurrence of cholinesterase in Papilionaceae, the family had the highest frequency of species reportedly having acetylcholine activity.

However definitive conclusion on association of the family of the plants with either presence or absence of neurotransmitter needs to be evaluated against their basal frequency in the native flora.

Number of species within a family exhibits various physiological actions. Several families show a high frequency of species possessing one or more physiological action with Cucurbitaceae, Papilionaceae and Solanaceae recording the highest number. Most of the families exhibited emetic effect (plants belonging to 28 families) while the least exhibited a nervine sedative property (plants belonging to 4 families). Similar to that reported in the plants used in treatment of neural disorder, it is found that epilepsy and neuralgia are the 2 major disorders against which maximum number of family (plants belonging to 23 and 26 family respectively) are used as curatives (Fig. 6).

Analysis of the plant parts used to cure neural disorder showed that most of the curatives originate from leaves, seeds, root, oil, fruit, flower and parallels the physiological action reported from them. Most of the plant parts had emetic, anodyne and anesthetic function in that order. Also most of the plant parts were used in treatment of epilepsy, neuralgia, vatavyadhi (Fig. 7). With respect to cholinesterase activity, over all the species analysed, leaves appear to be predominant source of cholinesterase followed by stem and root (Fig. 8).

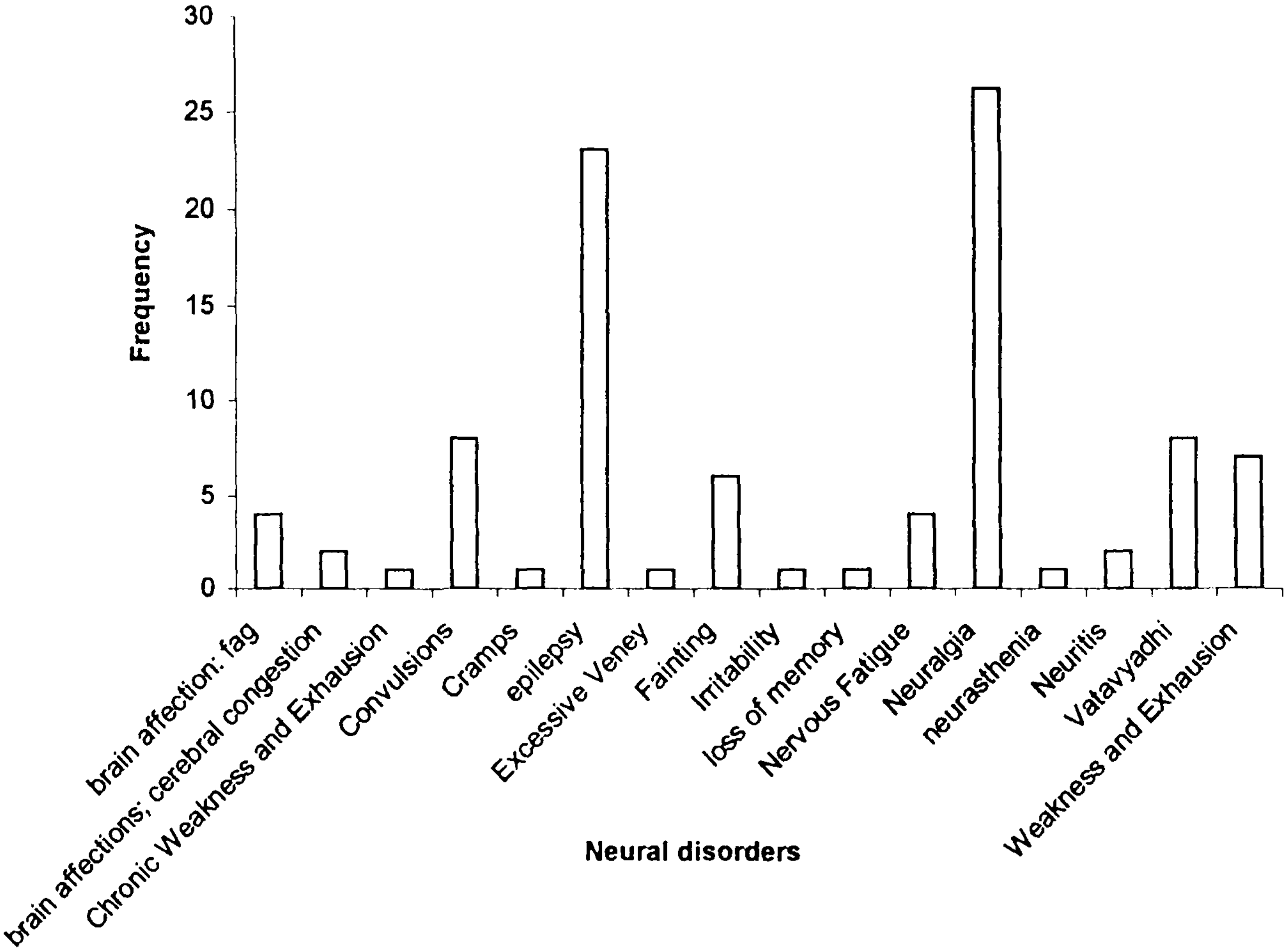


Fig. 6: Frequency of plant family used in treatment of neural disoders

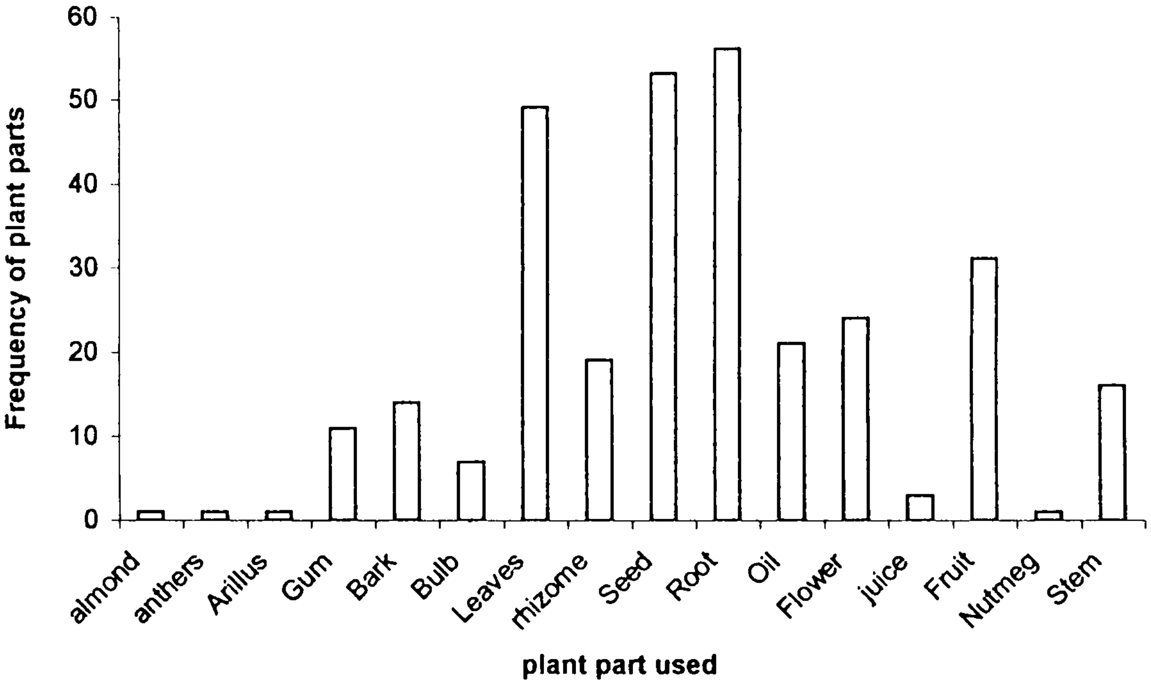


Fig. 7: Frequency of plant parts used to treat neural disorders.

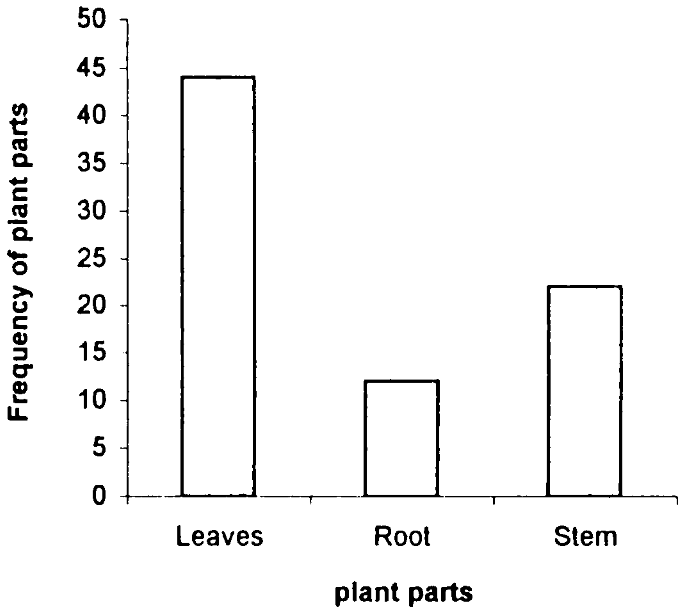


Fig. 8: Frequency of plant parts in which Cholinesterase activity has been reported.

results also paralleled that obtained for the physiological action; herbs, shrubs, trees, aquatic marshes, climbers in that order are most frequently used in rendering the various physiological actions (Fig. 9).

It is interesting to note that both in treatment of neural disorder and assay for the physiological action, the aqueous extract (around 56 and 51% respectively) was most commonly employed followed by powder or oil extraction (Fig. 10). This confirms well with the fact that most neurotransmitters are water-soluble.

Objective 2: Role of neurotransmitter in a few physiological processes in plants

The role of neurotransmitters in a few physiological processes namely pollen grain germination and tube growth and stomatal regulation was examined. Both of these processes involve the active exchange of ions across the cell membrane; for example in the pollen grain there is an active exchange of ions across the pollen cell membrane and the ambient medium during pollen grain germination and tube growth; in stomatal opening or closure the role of potassium ion exchange across the guard cell membrane is very well established. Because neurotransmitters in animal system primarily operate through the generation of action potential triggered by ion fluxes across membranes, it is expected that the model systems described here would be appropriate to examine the role of neurotransmitter in signal transmission. The results of these experiments are presented in this section.

Role of neurotransmitter in pollen grain germination and tube growth

The role of neurotransmitter in pollen grain germination and tube growth was examined using sunflower pollen grains. Pollen grains were treated with

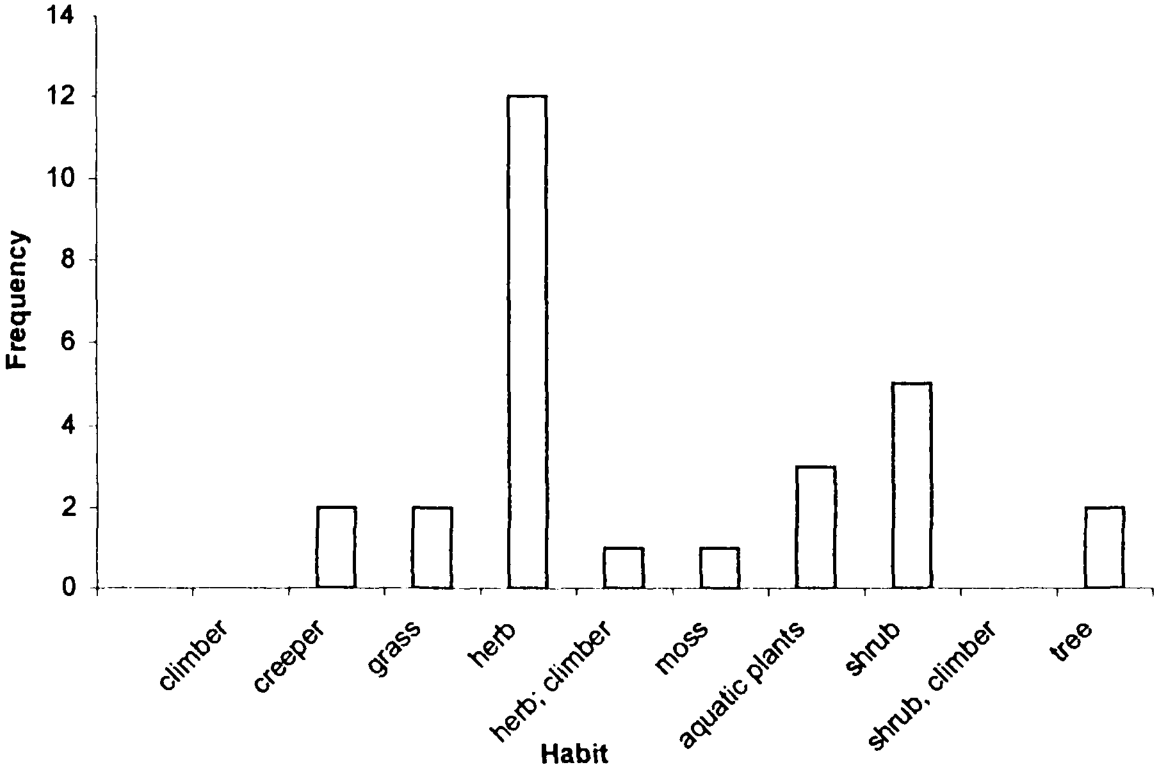


Fig. 9: Frequency of different habits of plants used in treatment of neural disorders.

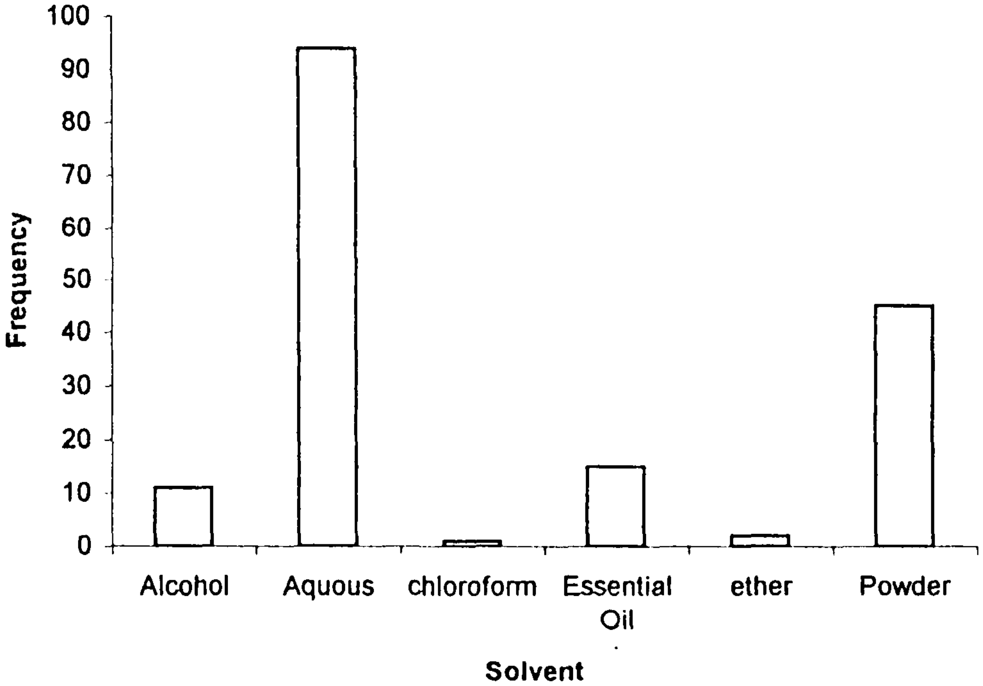


Fig. 10: Frequency of different solvent system used in extraction of curatives from plants used to treat neural disorders.

neurotransmitters and incubated in pollen germination media and the effect on pollen grain germination and tube growth monitored.

Effect of acetylcholine on pollen grain germination and tube growth

Pollen grains were treated with acetylcholine of varying concentration (0,1,10, 100, 500 ppm) and assayed for germination and tube length. Percentage germination and tube length increased with acetylcholine treatment upto a certain concentration and there after decreased indicating an optimal level of acetylchoine in this response. The maximum germination was found to occur at around 10ppm acetylcholine concentration (78% germination, which accounts for around 30% increase over control, Fig. 11). To examine the exact concentration at which maximum germination occurred ACh was tried at 0, 1, 3, 5, 7, 10, 12, 15 and 30 ppm (Fig. 12). Maximum germination of pollen grain was found to occur at 10 ppm ACh were in germination increased by 183% and tube length increased by 60% over control.

Effect of atropine (AT) an antagonist of ACh on pollen grain germination and tube growth

Pretreatment of pollen grains with different concentrations of atropine, an antagonist of acetylcholine, showed that the ACh (10ppm i.e. 55μM) induced increase in pollen grain germination was inhibited. The ACh induced increase in tube length was also inhibited by pretreatment with atropine (Fig. 13). Maximum of 80% decrease in germination occurred at 100μM atropine. ACh induced germination and tube growth was also inhibited by merely having the atropine in the pollen germination media with out pretreatment (Fig. 14). These results confirm the involvement of ACh as far as pollen germination and tube growth is concerned.

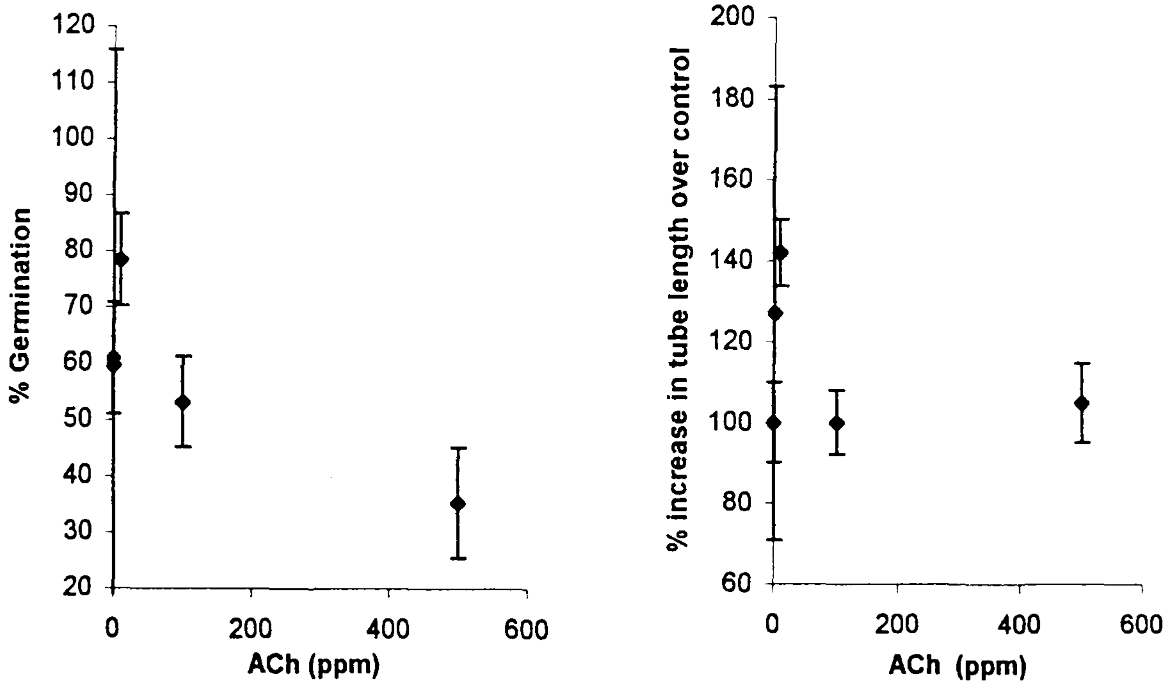


Fig. 11: Effect of acetylcholine on pollen grain germination and pollen tube growth.

2- way ANOVA, F- ratio significant at P< .05, .01, LSD(.05)= 6.670

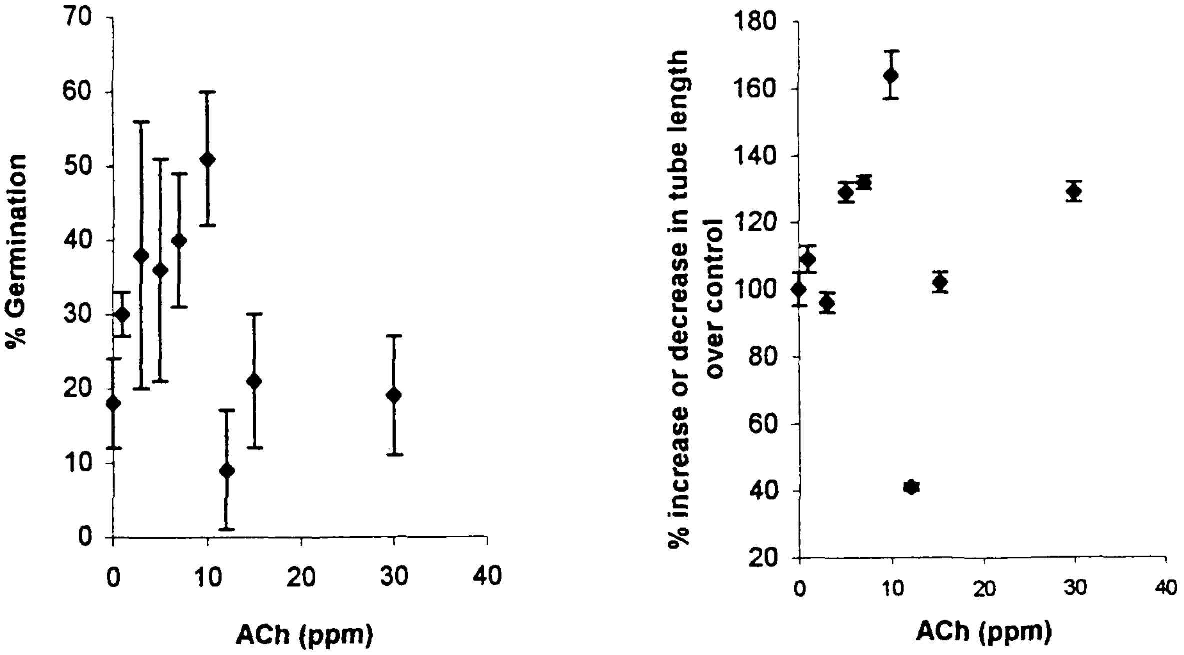


Fig. 12: Effect of varying concentration of acetylcholine on pollen grain germination and tube growth.

2- way ANOVA, F- ratio significant at P< .05, .01, LSD(.05)=7.146

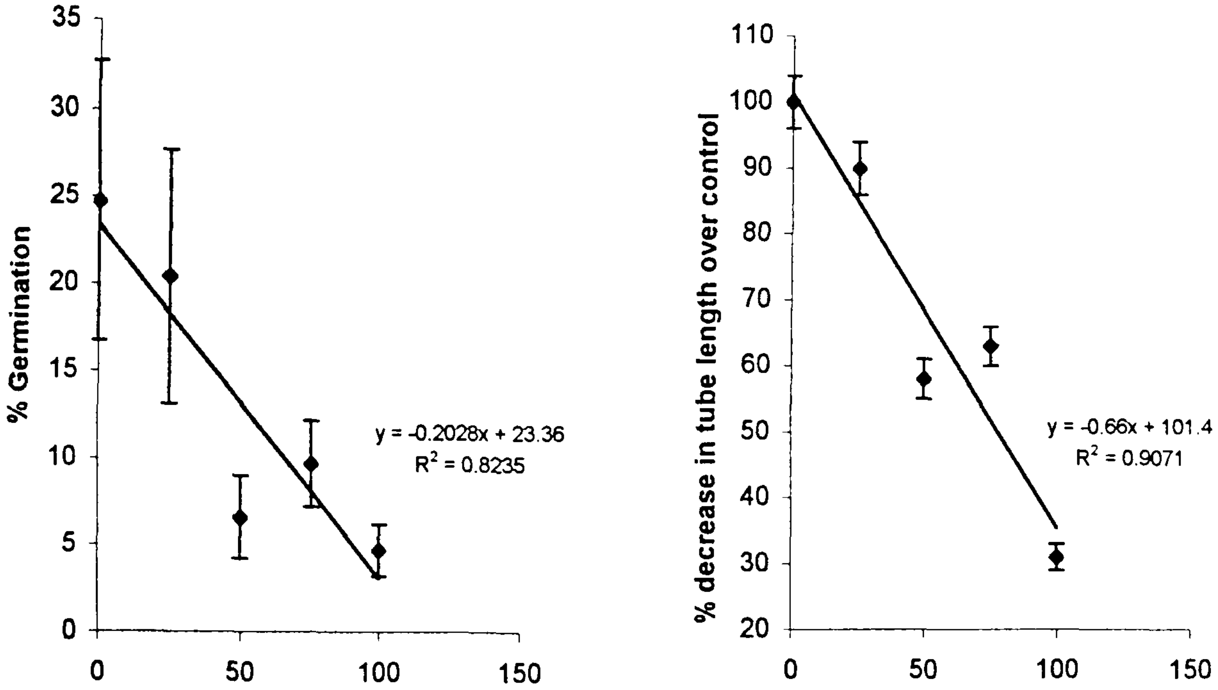


Fig. 13: Effect of atropine on acetylcholine induced pollen grain germination and tube growth (pollen grains were pretreated with atropine and then transferred to acetylcholine.

2- way ANOVA, F- ratio significant at $P< .05$, .01, LSD(.05)= 4.952

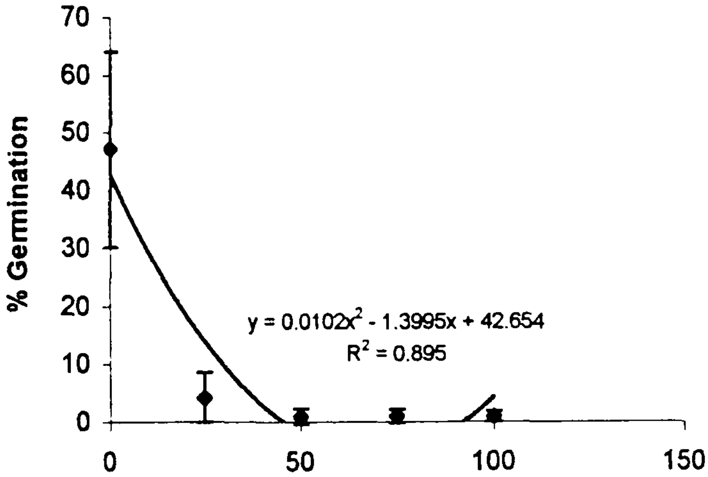


Fig. 14: Effect of atropine on acetylcholine induced pollen grain germination and tube growth (pollen grains were treated with atropine along with acetylcholine).

2- Way ANOVA, F- ratio significant at $P< .05$, .01, LSD(.05)= 6.865

Mode of action of ACh in enhancing pollen grain germination and tube growth

The mechanism underlying ACh mediated increase in pollen grain germination and tube growth was studied by examining the involvement of calcium in the model system. The rational for this study stems from the fact that calcium along with calmodulin has been reported to play an important role of second messengers in plants. Under the assumption that ACh could mediate stimulus response functions in plants, it is hypothesised that the actual carriers of stimulus could be initiated by the calcium ions. We examined the role of calcium ions in ACh dependent pollen grain germination and tube growth. Pollen grains were treated with EGTA, a calcium sequester and Lanthanum chloride a calcium transport inhibitor and were then incubated in ACh at different concentration. EGTA decreased the ACh induced percent germination by 30% at 25μM and tube growth but had no concentration dependent effect beyond 25μM (Fig. 15). In other words EGTA was able to reverse ACh induced germination in Sunflower pollen grains.

Pretreatment with EGTA resulted in significant decrease in germination and tube length in a dose dependent manner; however such dose dependent manner was not apparent for pollen tube length. The germination decreased by 43% at 25μM EGTA and maximum of 72% reduction occurred at 75μM EGTA compared to germination in presence of acetylcholine alone (Fig. 16). These results suggest that ACh induced increase in percentage germination might be mediated by the release of cell membrane bound calcium which then could lead to a cascade of metabolic events resulting in enhancement of pollen grain germination and tube growth.

Lanthanum chloride also resulted in decrease in ACh induced germination by 92% and tube growth by 64% at 25μM. The percentage decrease in germination and tube growth was quite substantive even at 25μM of

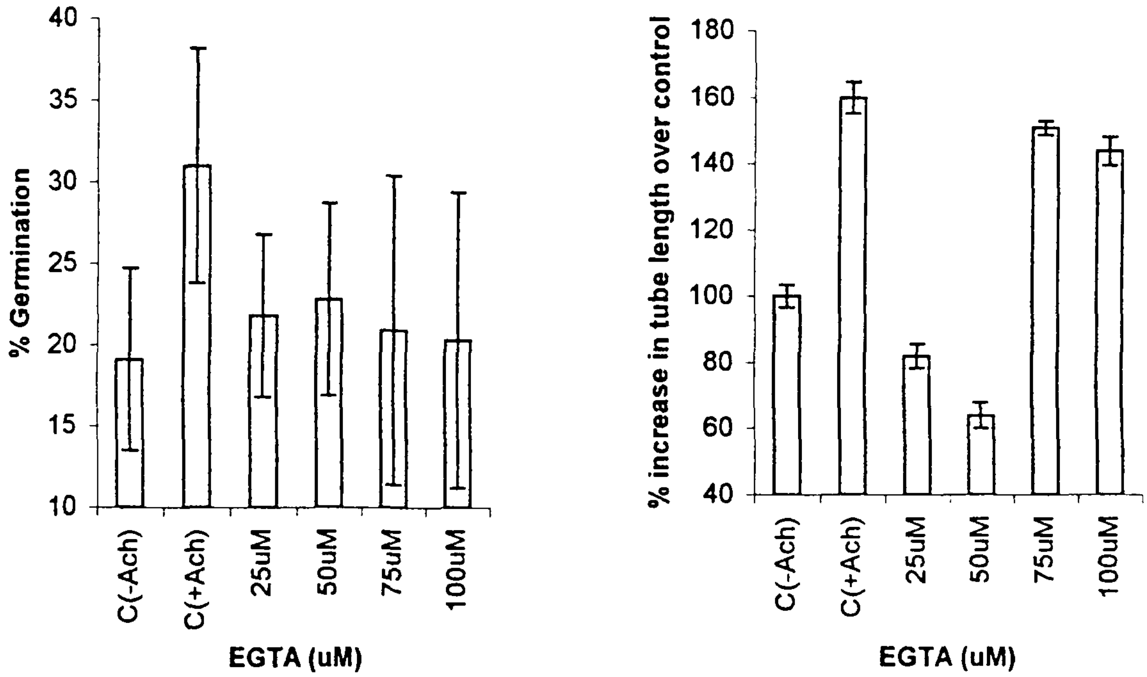


Fig. 15: Effect of EGTA on acetylcholine induced pollen grain germination and tube growth (pollen grains were treated with acetylcholine along with different concentrations of EGTA in the pollen grain germination medium).

2- way ANOVA, F- ratio significant at $P < .05$, LSD(.05)=6.838

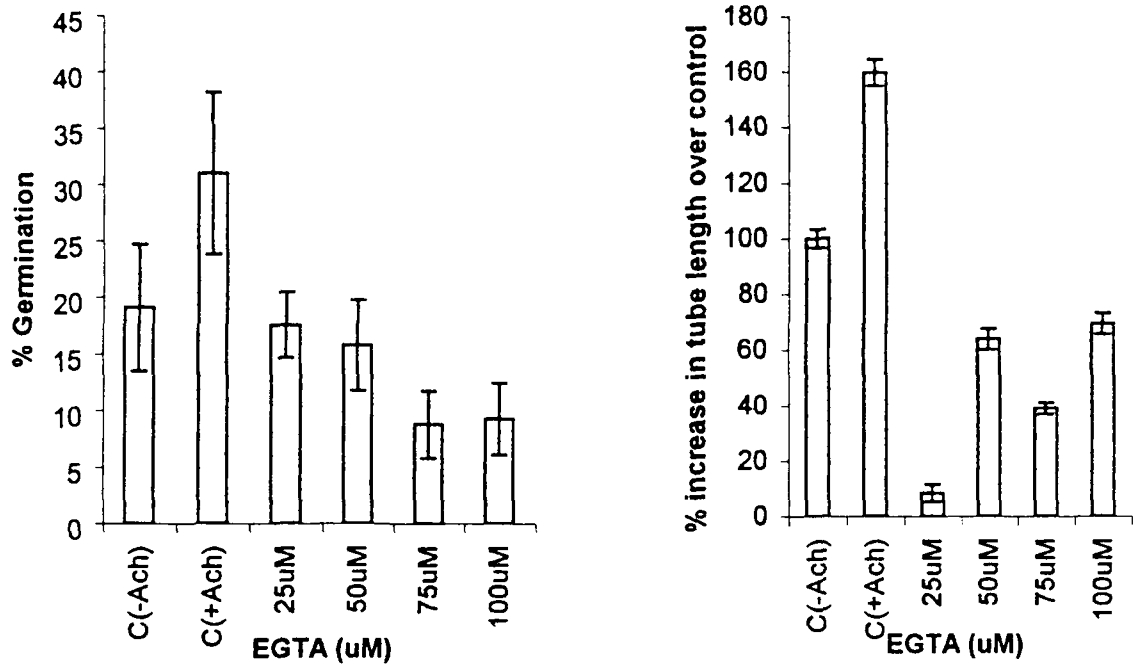


Fig. 16: Effect of EGTA pretreatment on acetylcholine induced pollen grain germination and tube growth (pollen grains were pretreated with EGTA and then transferred to acetylcholine.

2- way ANOVA, F- ratio significant at $P < .05$, .01, LSD(.05)= 4.315

lanthanum chloride (Fig. 17). These results also confirm the possible mediation of calcium in ACh induced germination and pollen tube growth.

Role of other neurotransmitters in pollen grain germination

The role of a number of other neurotransmitters viz. Dopa, glutamate, GABA, adrenaline in pollen grain germination and tube growth was examined using the model system of Sunflower. Pollen grains in presence of glutamate showed a significantly less germination (95% decrease over control, Fig. 18) and thus acted in a manner opposite to acetylcholine. Treatment with adrenaline yielded a parabolic relationship with pollen grain germination with 25 and 50μM (77 and 71% respectively) showing maximum germination. Concentrations above these were found to be inhibitory (Fig. 19). Adrenaline had no effect on pollen tube growth. Treating pollen grains with GABA resulted in decrease in pollen germination (maximum of 44% decrease in germination occurred at 75μM GABA) and had no effect on pollen tube growth (Fig. 20). On the other hand treatment with dopa was known to enhance germination but had no effect on tube length. Maximum of 60% germination was observed at 50μM concentration (Fig. 21). In conclusion it appears that neurotransmitters as a group have different and opposite effect on pollen grain germination. ACh, adrenaline and Dopa seem to increase germination while glutamate and GABA were found to have an inhibitory effect. Effect of neurotransmitters on pollen tube growth was not as forthcoming.

Role of neurotransmitters in stomatal regulation

We examined the role of neurotransmitters in the opening and closure of stomata in epidermal peels of *Commelina*. The stomatal bioassay was standardized using KCl-$CaCl_2$ buffer. The results show that with increase in concentration of KCl there is a clear increase in opening but the pattern of increase differs with progression of time. At 1 hr of incubation in the buffer, the

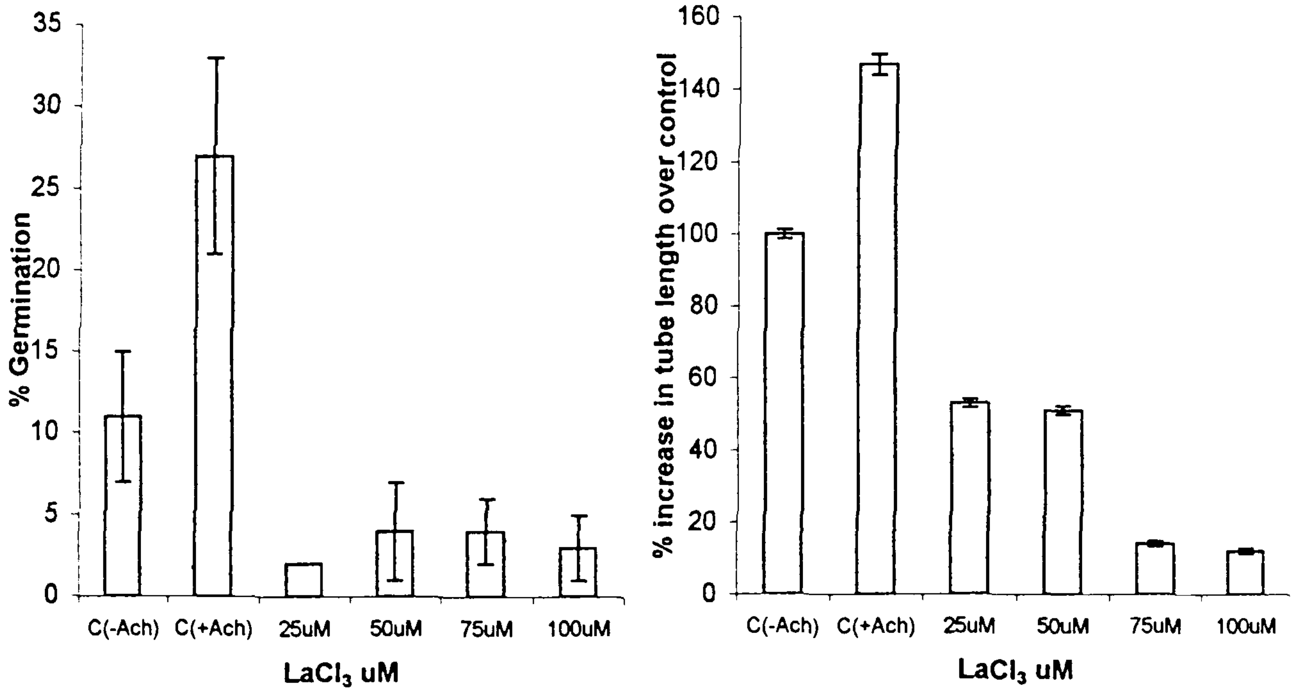


Fig. 17: Effect of Lanthanum chloride pretreatment on ACh induced pollen grain germnation and tube growth (pollen grains were pretreated with $LaCl_3$ and then transfer to acetylcholine.

2- way ANOVA, F- ratio significant at $P< .05, .01$, LSD(.05)= 3.014

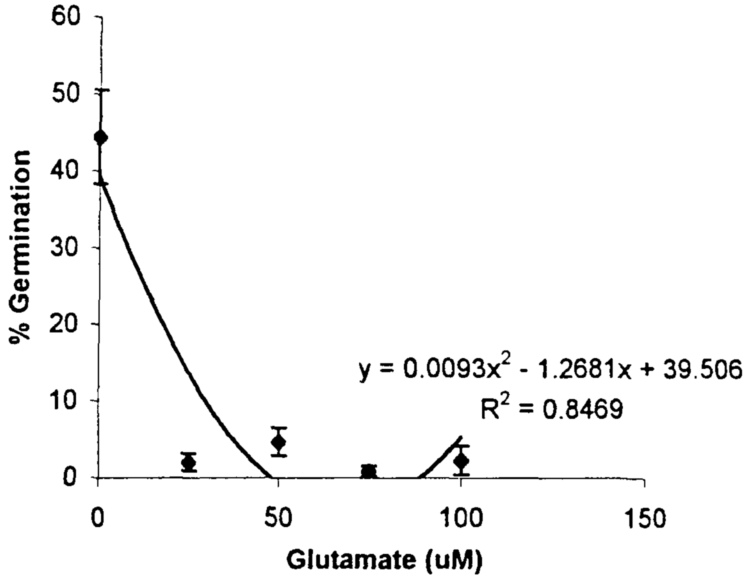


Fig. 18: Effect of glutamate on pollen grain germination.

2- way ANOVA, F- ratio significant at P< .05, .01, LSD(.05)= 4.611

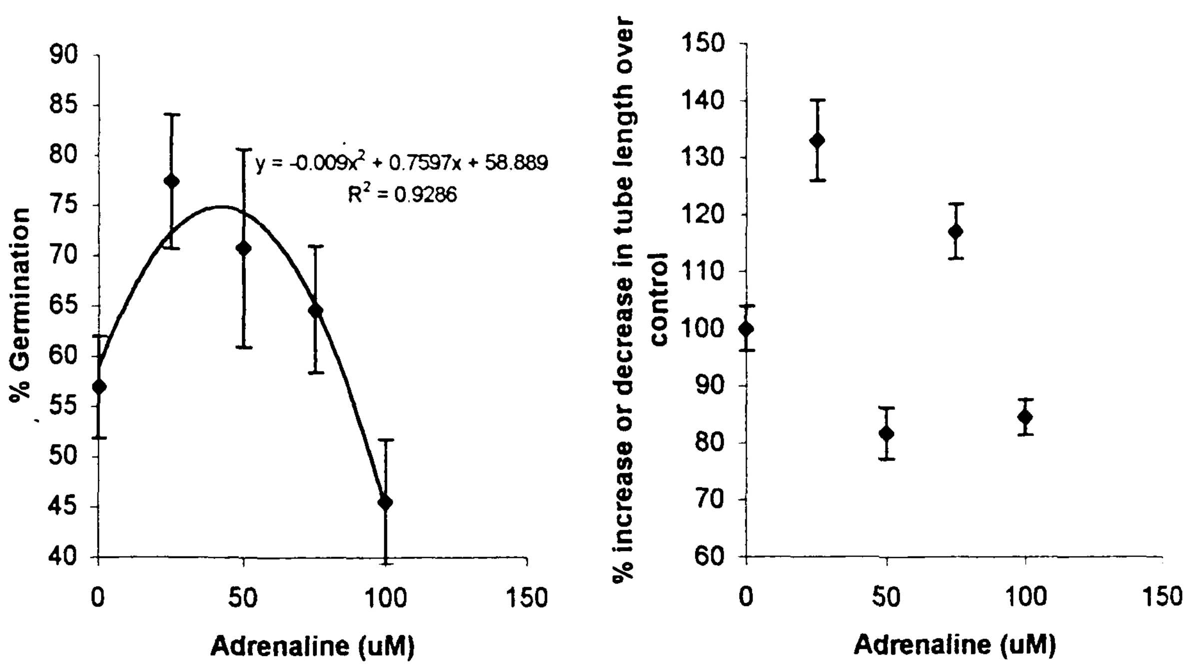


Fig. 19: Effect of adrenaline on pollen grain germination and tube growth.

2- way ANOVA, F- ratio significant at P< .05, .01, LSD(.05)=6.35

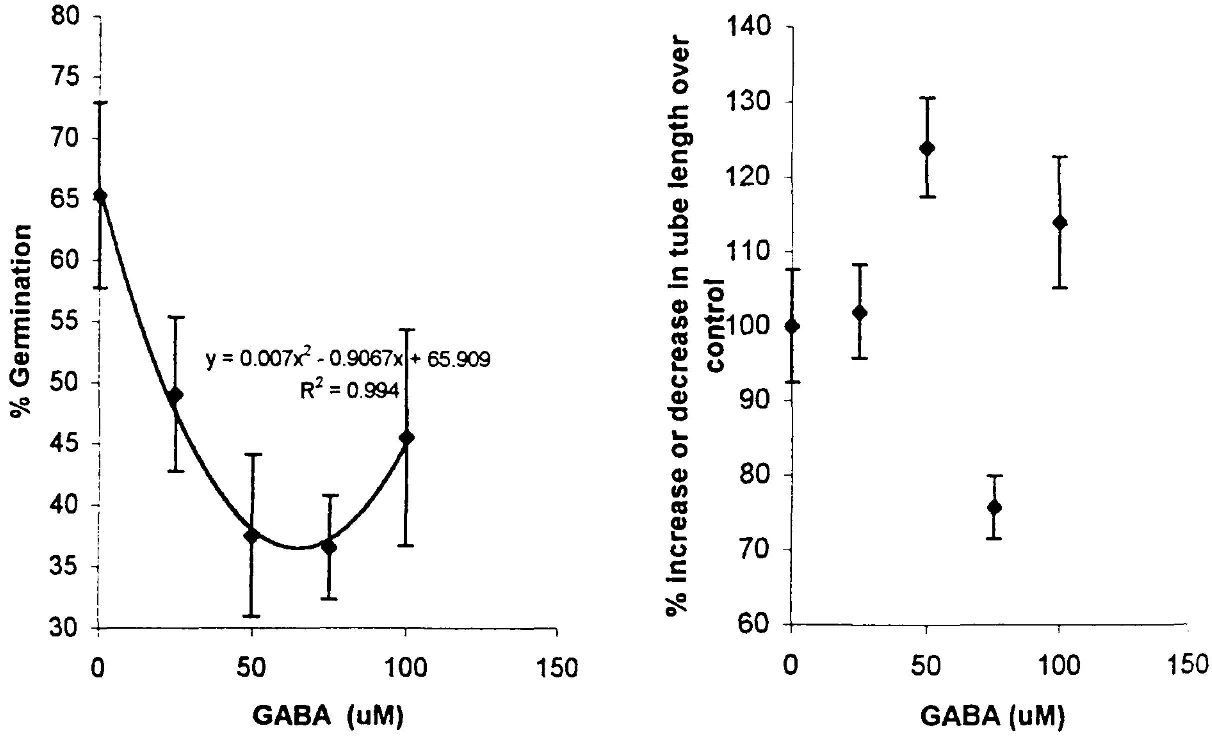


Fig. 20: Effect of GABA on pollen grain germination and tube growth.

2- way ANOVA, F- ratio significant at P< .05, .01,

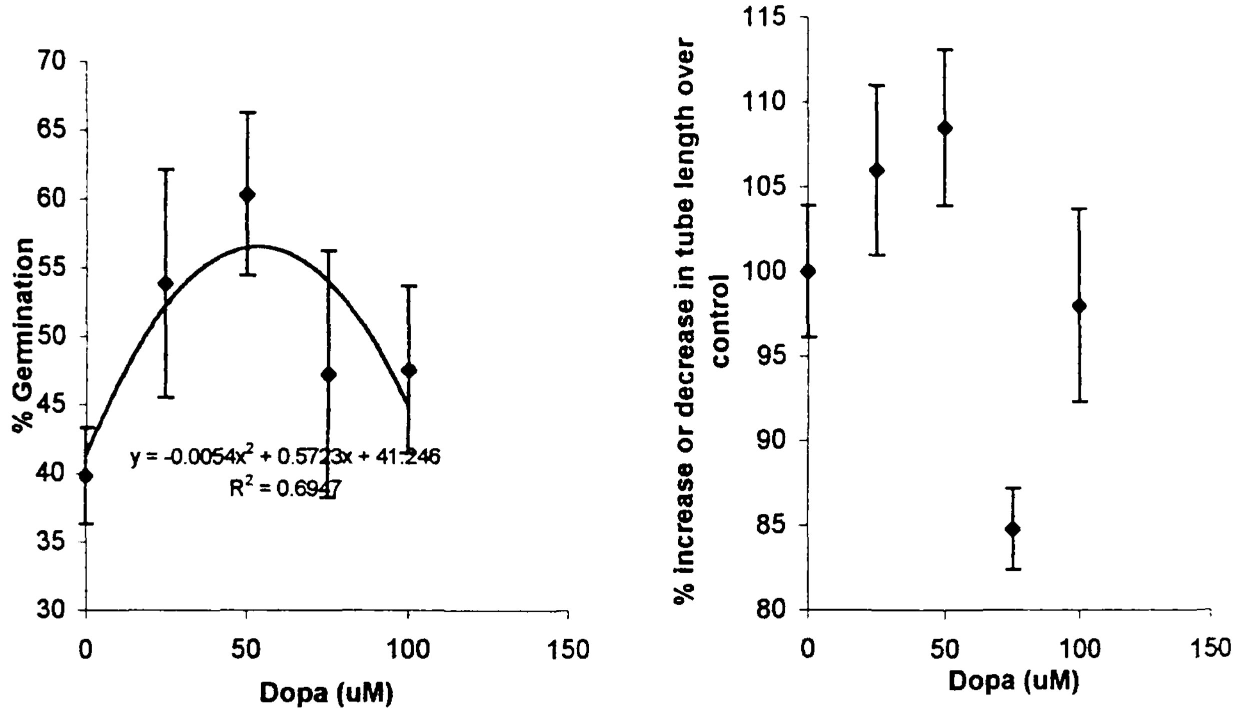


Fig. 21: Effect of dopa on pollen grain germination and tube

2- way ANOVA, F- ratio, significant at P< .05, .01, LSD(.05)= 7.335

Commelina epidermal peels showed a clear parabolic relation, with maximum opening (14 to 15μm) at 100 – 150mM KCl. At 2 hr after incubation the opening of the stomata reached a asymptote (around 11μm) at about 150mM of KCl (Fig. 22). Based on these studies, further experiments involving neurotransmitters were conducted on epidermal peels incubated in 100mM KCl under optimum light conditions.

Role of acetylcholine on stomatal opening at cellular level

To examine the role of Acetylcholine in stomatal regulation, the epidermal peels of *Commelina* were incubated in different concentration of ACh (0, 1, 10, 50, 100. 200, 300, 400, 500, 700 and 1000μM) along with KCl- $CaCl_2$ buffer. Observations recorded at 1 hr after incubation indicated that ACh was inhibitory for stomatal opening. The opening decreased by 40% over control in presence of 200μM acetylcholine after 1 hr of incubation. Significant closure of stomata occurred in presence of ACh in a dose dependent manner. ACh was found to increase stomatal closure. Drastic reduction in opening occurred until 400μM (70% reduction over control) in a dose dependent manner; concentrations above 400μM did not show dose dependent effect (Fig. 23).

Evidence for the involvement of ACh in stomatal regulation was also provided by the use of Atropine. Peels were pretreated with Atropine before incubation with ACh. Atropine reversed the ACh induced closure. Atropine was found to enhance stomatal opening in a dose dependent manner (Fig. 24). At 100μM the opening was restored back to that obtained under control conditions.

To examine the role of ACh in stomatal regulation via Calcium signaling, the peels were treated with EGTA (Calcium chelator) of varied concentration. No significant effect was observed after 1 hr incubation. However after 2 hr, there was a clear increase in the opening of stomata (opening was restored to the control levels at 100μM EGTA) indicating that ACh induced stomatal closure occurred via Calcium (secondary messenger) (Fig. 25) (Plate 1).

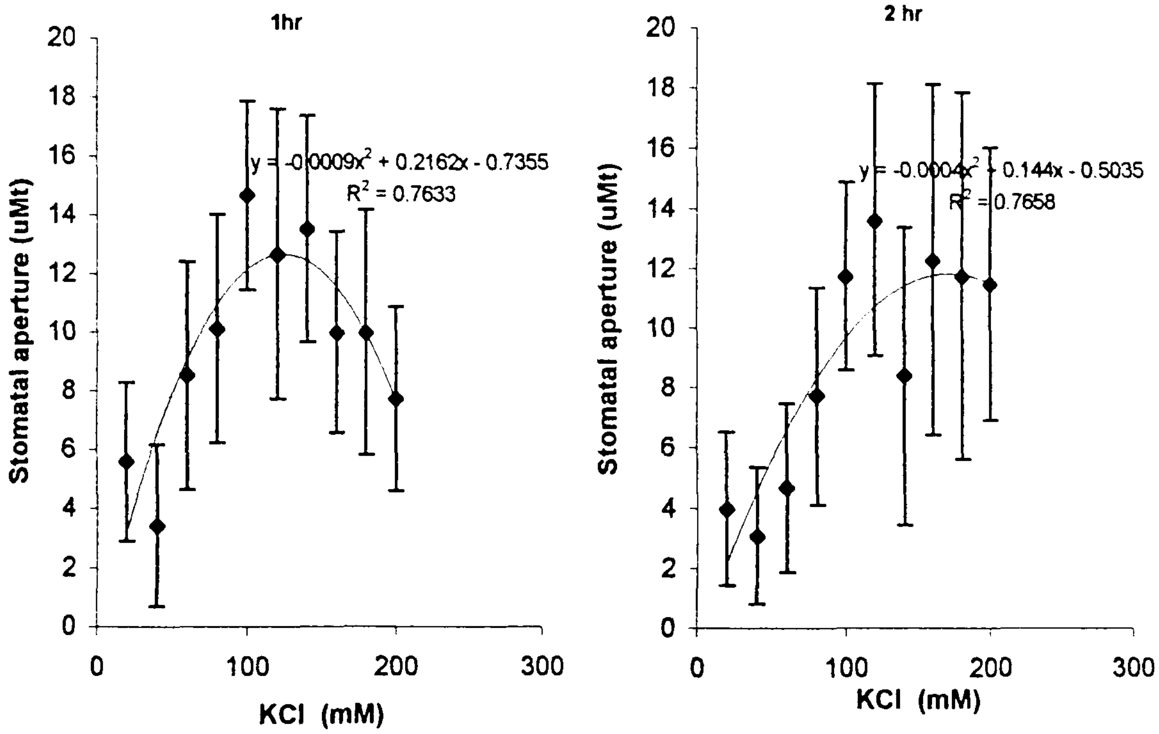


Fig. 22: Effect of KCl on stomatal opening in *Commelina sps* epidermal peels.

2- way ANOVA, F- ratio significant at P< .05, .01 both after 1hr and 2hr incubation, LSD(.05)3.590

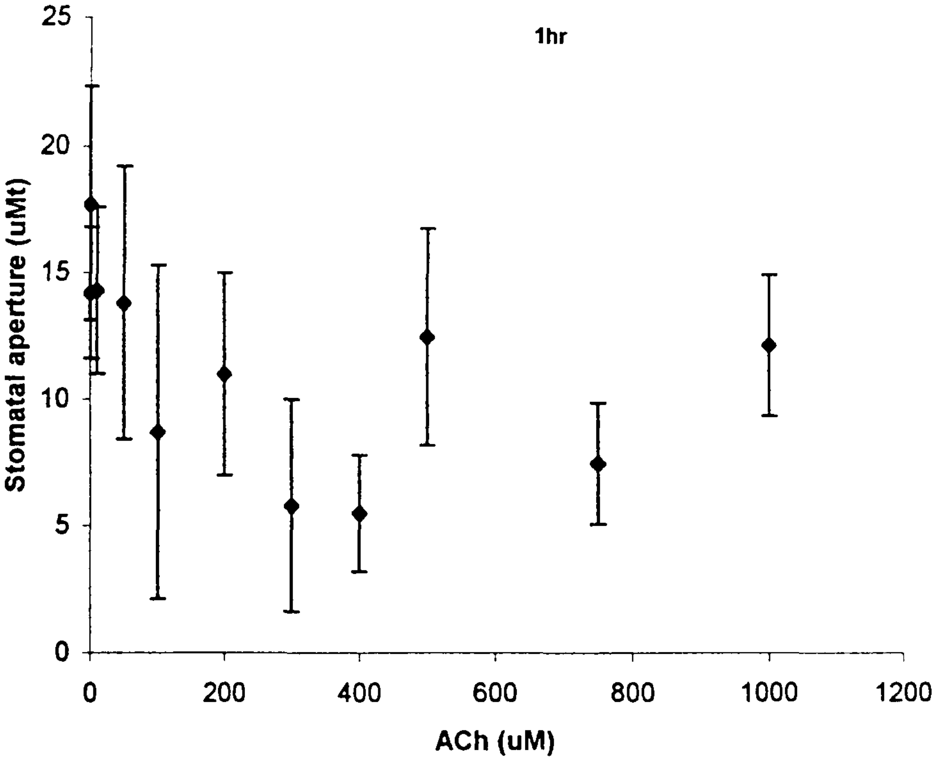


Fig. 23: Effect of Acetylcholine on stomatal opening.

2- way ANOVA, F- ratio significant at $P < .05, .01$, LSD(.05)=.83

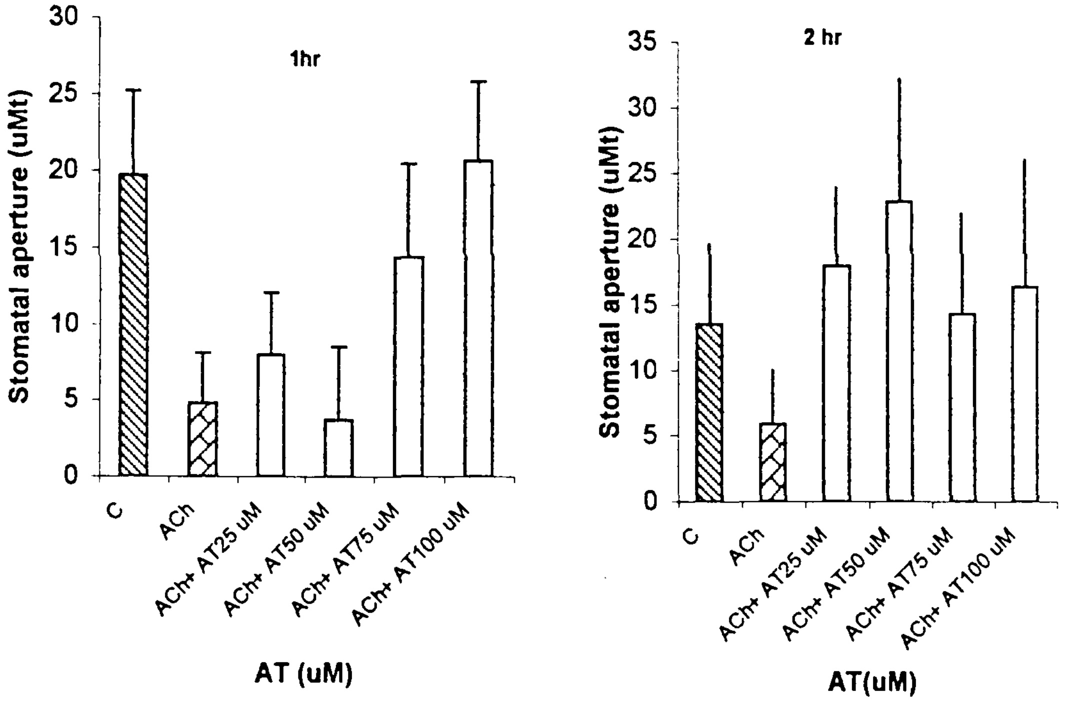


Fig. 24: Effect of Atropine on acetylcholine induced stomatal closure.

2- way ANOVA F-ratio significant at PLANTS< .05, .01 both after 1 and 2hr of incubation, LSD(.05)=6.450

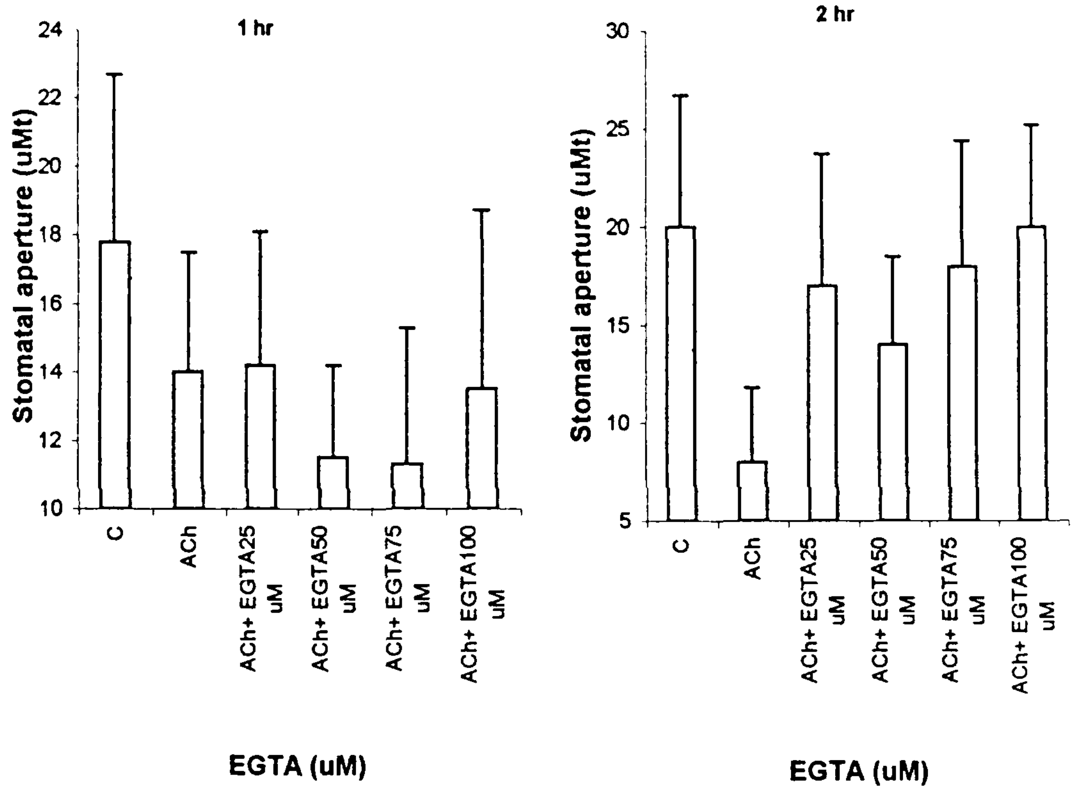


Fig. 25: Effect of EGTA pretreatment of epidermal peels on Acetylcholine induced stomatal closure.

2- way ANOVA, F-ratio significant at PLANTS< .05, .01 both after 1 and 2hr of incubation, LSD(.05)= 1.319

1. Control (only buffer)
2. Control (Buffer+ ACh 200μM)
3. ACh 200μM + 50μM AT
4. ACh 200μM + 100μM AT
5. ACh 200μM + 25μM EGTA
6. ACh 200μM + 50μM EGTA
7. ACh 200μM + 75μM EGTA
8. ACh 200μM + 100μM EGTA

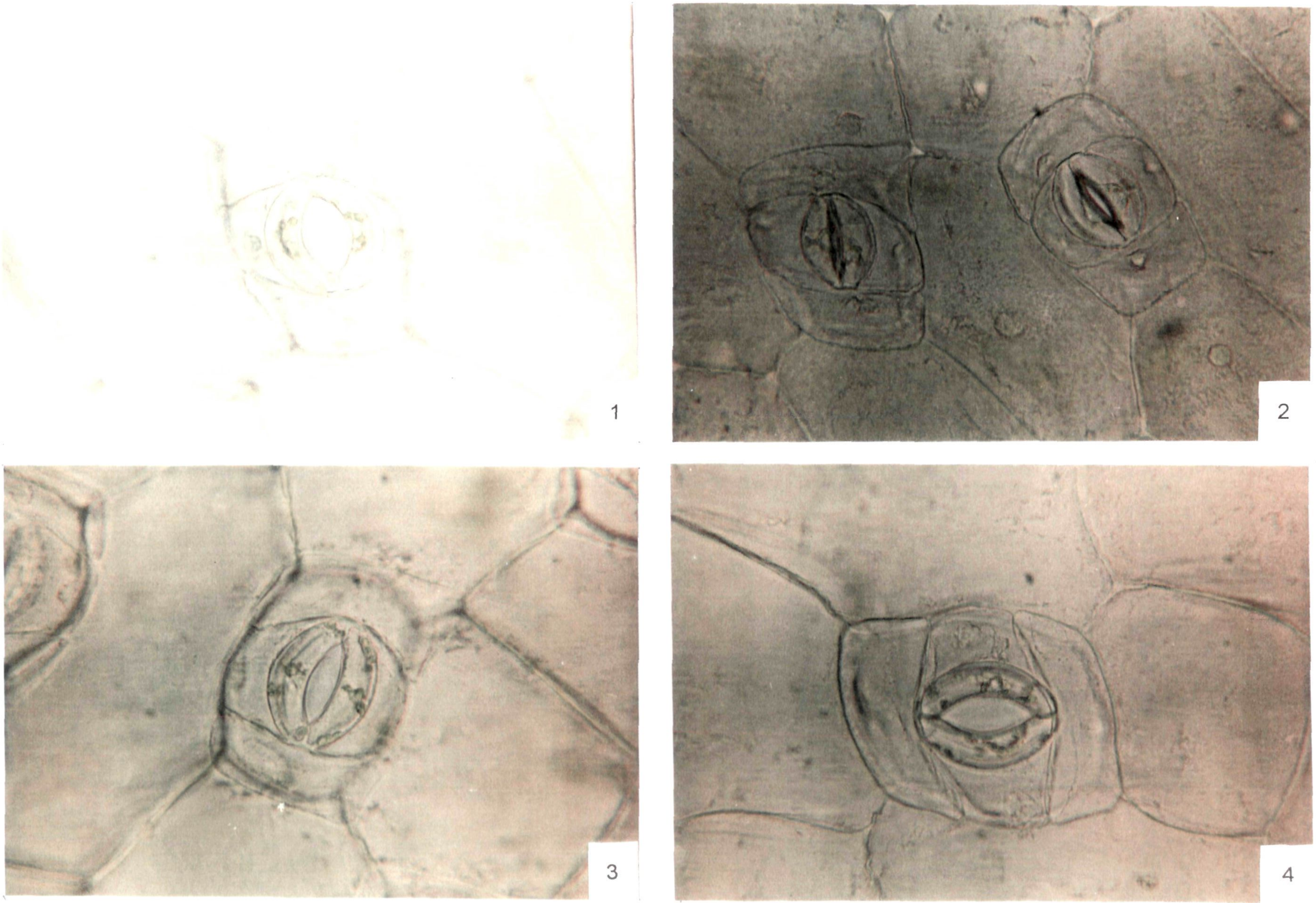


Plate 1. Effect of Acetylcholine, its inhibitor atropine and calcium chelator

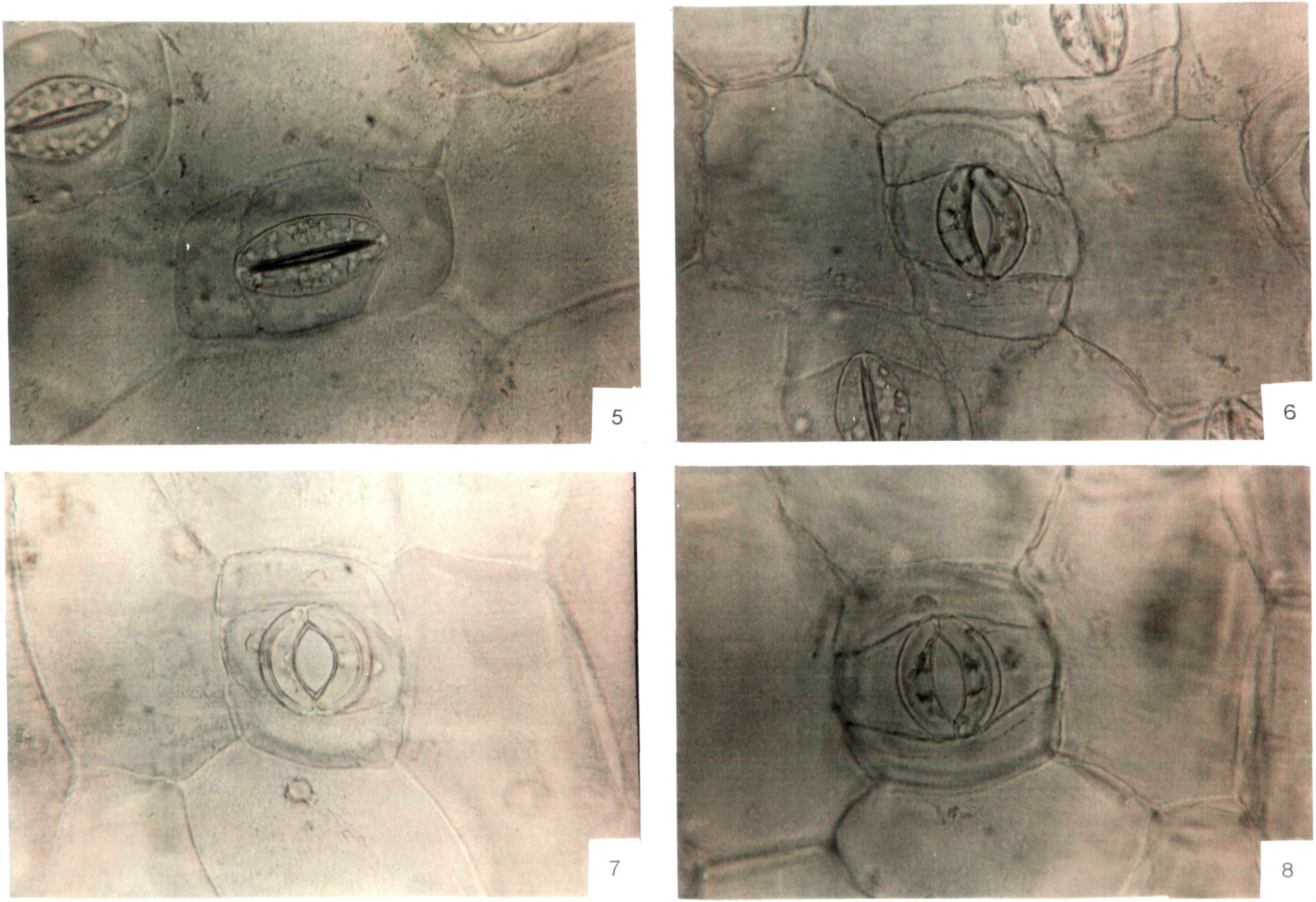


Plate 1: Effect of Acetylcholine, its inhibitor atropine and calcium chelator EGTA on stomatal opening.

The above results indicate that acetylcholine may play a significant role in stomatal regulation by inducing stomatal closure. These results are confirmed by the use of atropine, which reverses the ACh induced stomatal closure. The results also suggest that similar to the mediation of calcium in pollen grain germination the stomatal closure by ACh might also be mediated by calcium.

Role of neurotransmitters on stomatal regulation at single leaf level

Experiments were conducted to examine the role of neurotransmitters on stomatal regulation at single leaf level. Petioles of Sunflower leaves were pretreated with ACh (100μM) for 6 hr before transferring to a known volume of water and monitored for transpirational loss after 3 hr. It was found that ACh did not significantly effect stomatal closure at single leaf level. Even incubation with ACh for 3 hr did not have any significant effect. After 24 hr, there was 25% reduced water loss in ACh treated leaves, but the reduction was not significant (Fig. 26).

Experiments were conducted to monitor the rate of water loss during the initial 6 hr of treatment with neurotransmitters (ACh). It was observed that with time the water loss increased; however in presence of 50 and 100μM ACh the loss of water was less compared to the control (Fig. 27).

Treatment of leaves with GABA showed a reduction in the rate of water loss, indicating a role of GABA in regulating stomata. However, other neurotransmitters such as Dopa did not have any effect on stomatal regulation (Fig. 28).

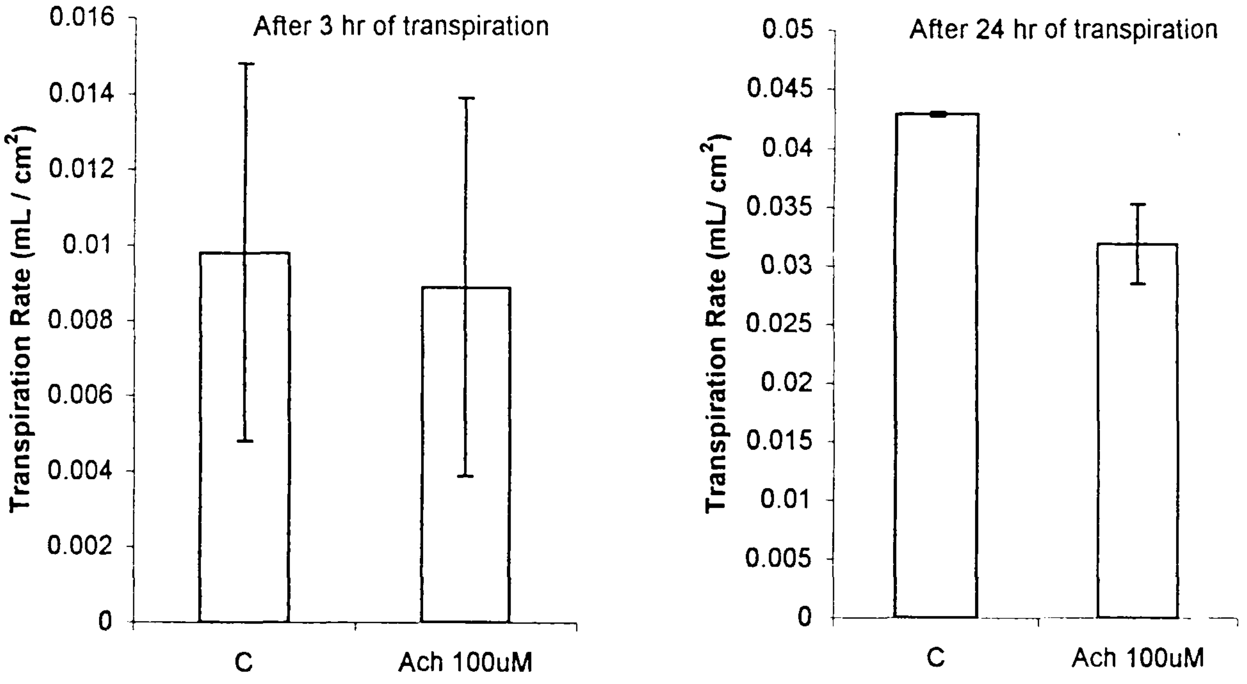


Fig. 26: Effect of Acetylcholine (3 hr pretreatment) on transpiration rate of Sunflower leaves.

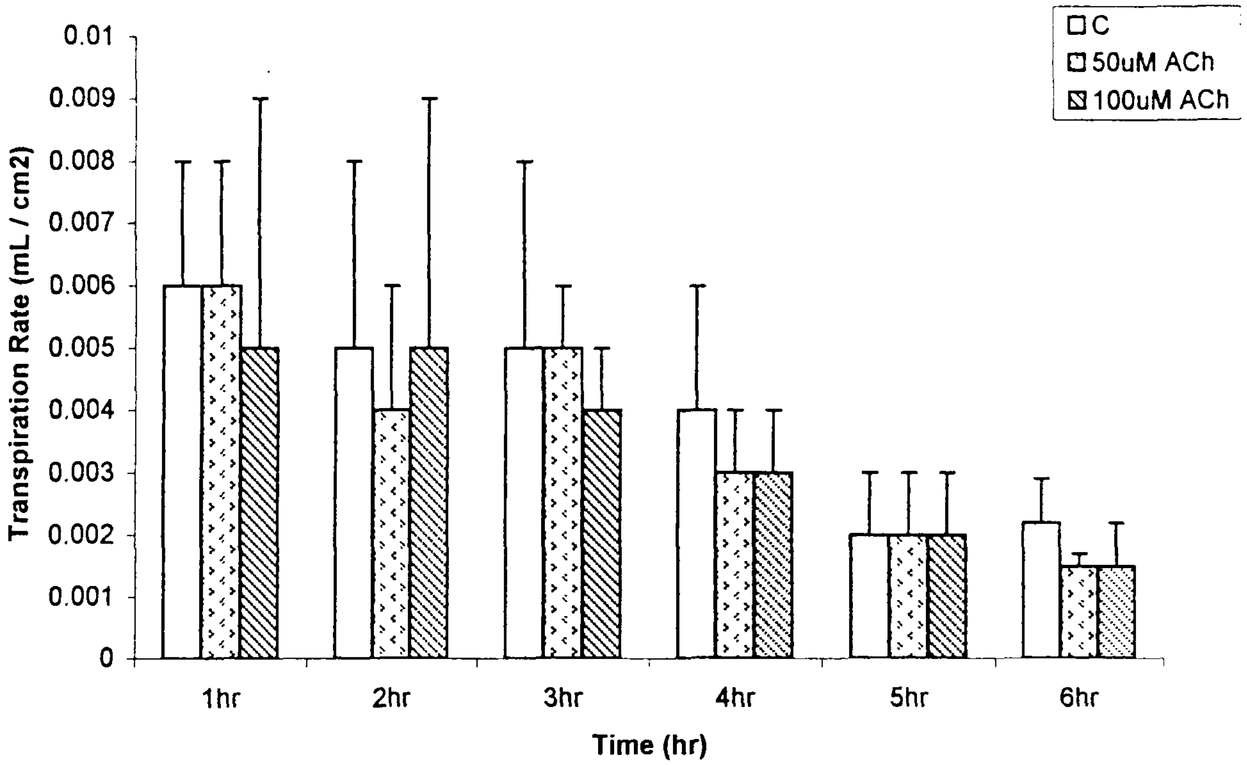


Fig. 27: Effect of Acetylcholine on transpiration rate during 6 hr of treatment.

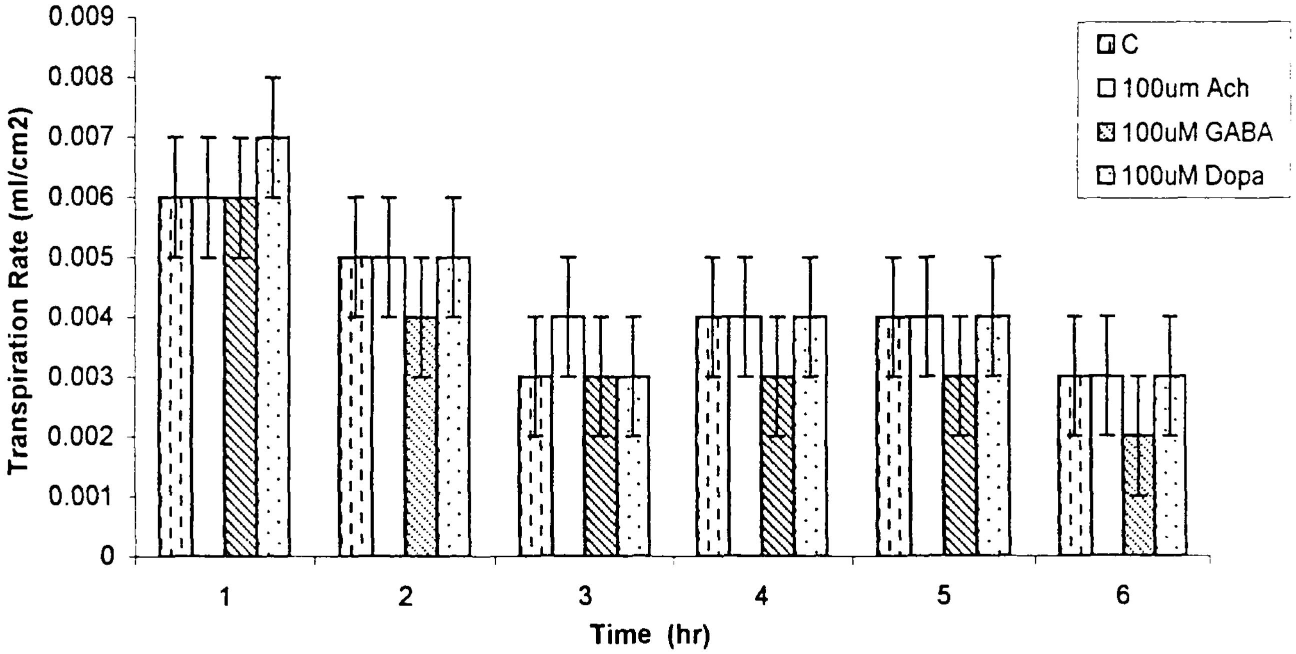


Fig. 28: Effect of Acetylcholine, GABA, Dopa on transpiration rate during 6 hr treatment.

Objective 3: Role of neurotransmitters in tactile motion in plants

The role of neurotransmitters in the tactile motion in plants was studied using two model systems viz. tendril of cucurbits and parasitic stem of cuscutta. In both these systems tactile motion is fundamental to the eventual success of the plants either for passive support (anchoring) or for retrieval of nutrients. Thus both systems should have been selected to evolve highly sensitive tactile motion behavior or response to stimuli. our hypothesis is that such systems would be ideal to examine the role of neurotransmitters in the plants. In these series of experiment the role of ACh and other neurotransmitters in the tactile motion of the two-model system have been studied.

Role of neurotransmitters in tendril movement

Effect of ACh on tendril coiling

Tendrils of Cucurbitaceae plants were pretreated with ACh and the rate of coiling in a free-floating medium was monitored. Compared to the control, the rate of coiling was faster and higher when tendrils were treated with ACh (Fig. 29a). Thus at the end of thirty minutes of incubation compared to about 20% of coiling in control tendrils, those treated with 1000μM ACh exhibited about 60% coiling. The results were confirmed in separate experiments (Fig. 29b).

Atropine one of the inhibitors of ACh receptors did not inhibit the ACh Induced coiling of tendrils (Fig. 30) at any of the concentration used. It is likely that the induction of coiling by ACh is mediated by an independent receptor, which is not inhibited by atropine. For example it is reported that ACh has two receptors sites viz. nicotinic and muscarnic AChR. Atropine is known to be active only against muscarnic AChR site. Intriguingly atropine alone also increased the rate of coiling compared to that in control, confirming that the

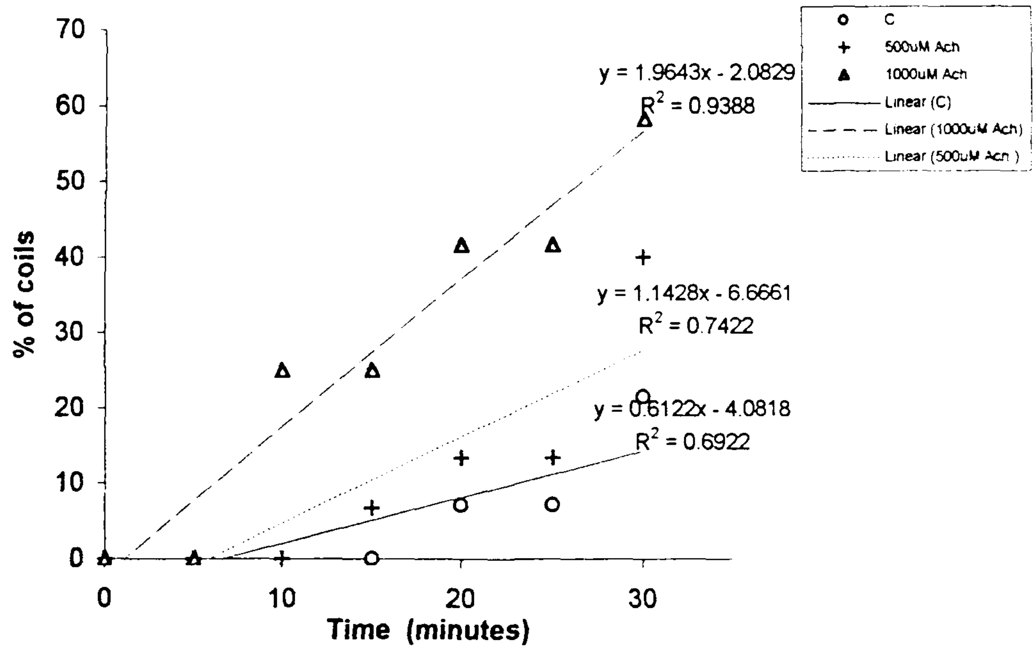


Fig. 29(a): Effect of Acetylcholine on coiling of tendrils (I expt)

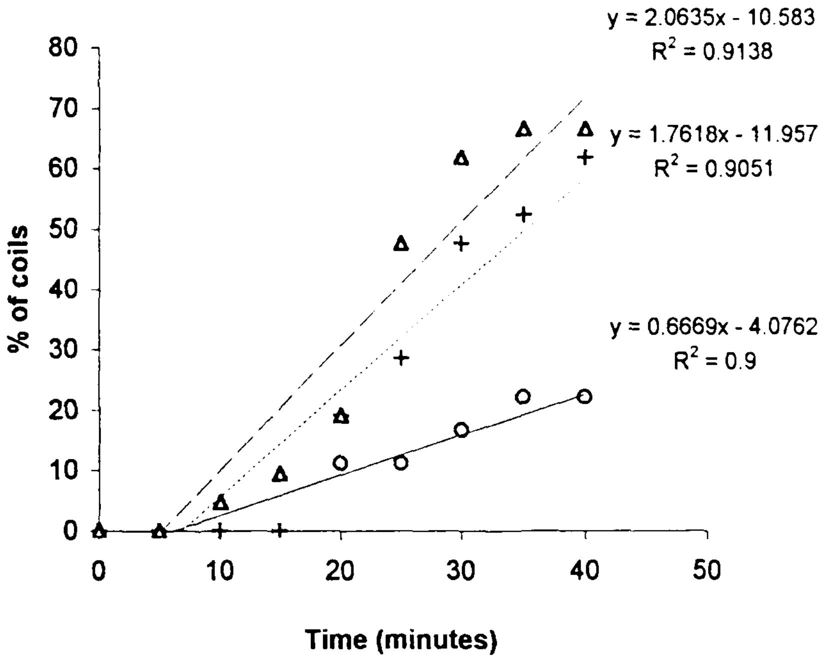


Fig. 29(b): Effect of Acetylcholine on coiling of tendrils (II expt)

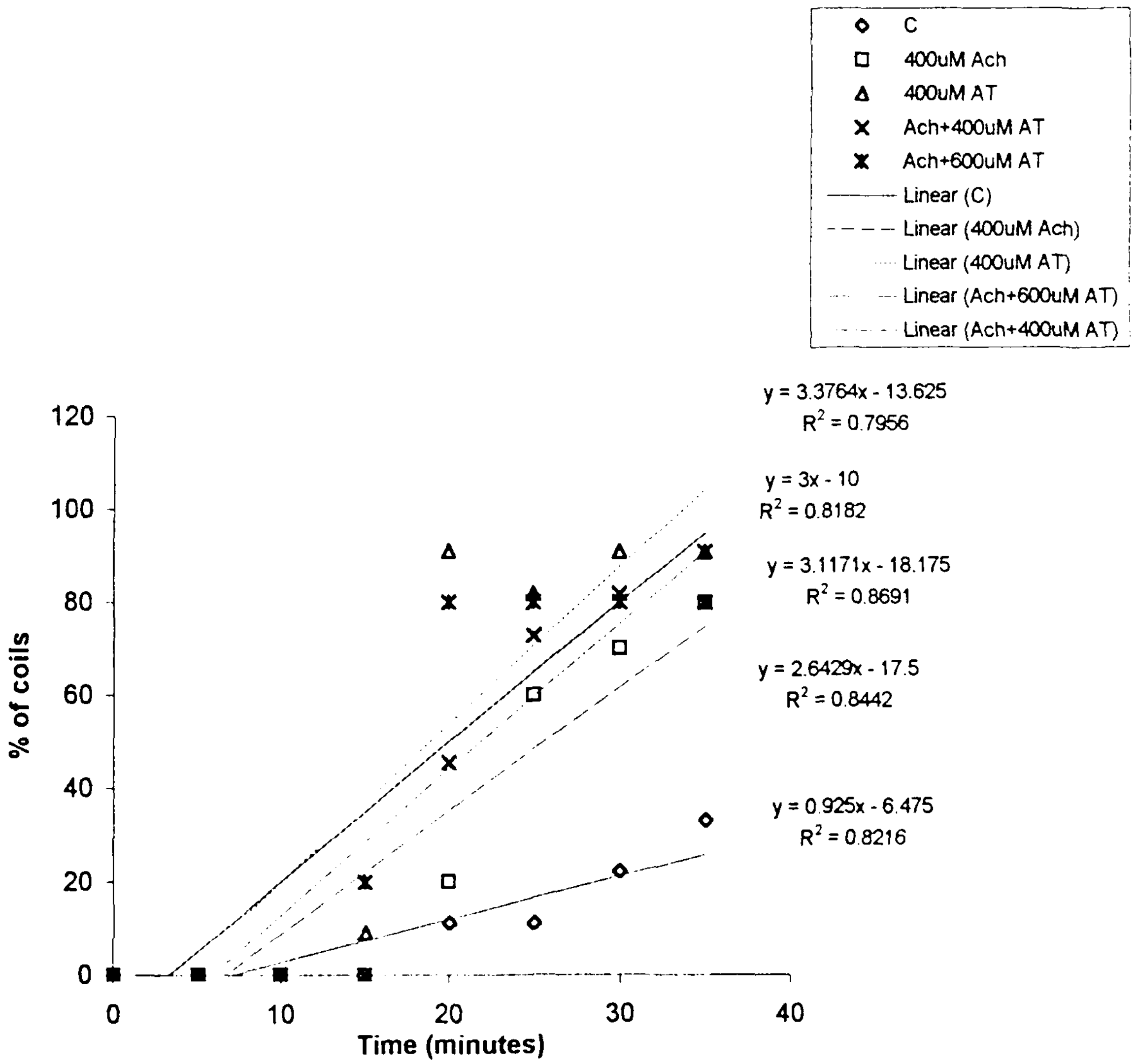


Fig. 30: Effect of Atropine on acetylcholine induced coiling.

receptor blocked by atropine may not be responsible for ACh induced coiling of tendrils.

Effect of glutamate on coiling

Glutamate, another neurotransmitter, showed an increase in the rate of coiling compared to control at 25μM (90% coiling compared to control) at 30 min. At concentrations beyond this, glutamate did not increase the rate of coiling compared to that of the control (Fig. 31).

Role of neurotransmitters in preference of host by Cuscutta

Effect of ACh on preference of host

The acceptance or rejection by Cuscutta stems of live and dead support respectively as influenced by ACh was examined. Cuscutta stems were anchored to dead support consisting of dead wood material and movement away (rejection) and movement towards (acceptance) was recorded. Distinctly the rate of rejection (moving away) was faster in presence of ACh compared to that in control (Fig. 32). 75% of the ACh treated stems rejected (45^0) the dead support where as there was no rejection in the untreated ones at 45 min. after incubation. In fact the rejection response as measured by the angle moved away ($45^0/90^0$) from support clearly indicated a rejection response mediated by ACh.

GABA, another neurotransmitter, also showed a marginal increase in the rejection of dead support with time compared to the control (Fig. 33). Dopa and glutamate were two other neurotransmitters examined for the rejection response. In presence of 1000μM Dopa there was 100% rejection (45^0) after 60 min. of incubation compared to control with 66% rejection. In presence of glutamate (500 or 1000μM) there was 83% rejection (45^0) compared to control

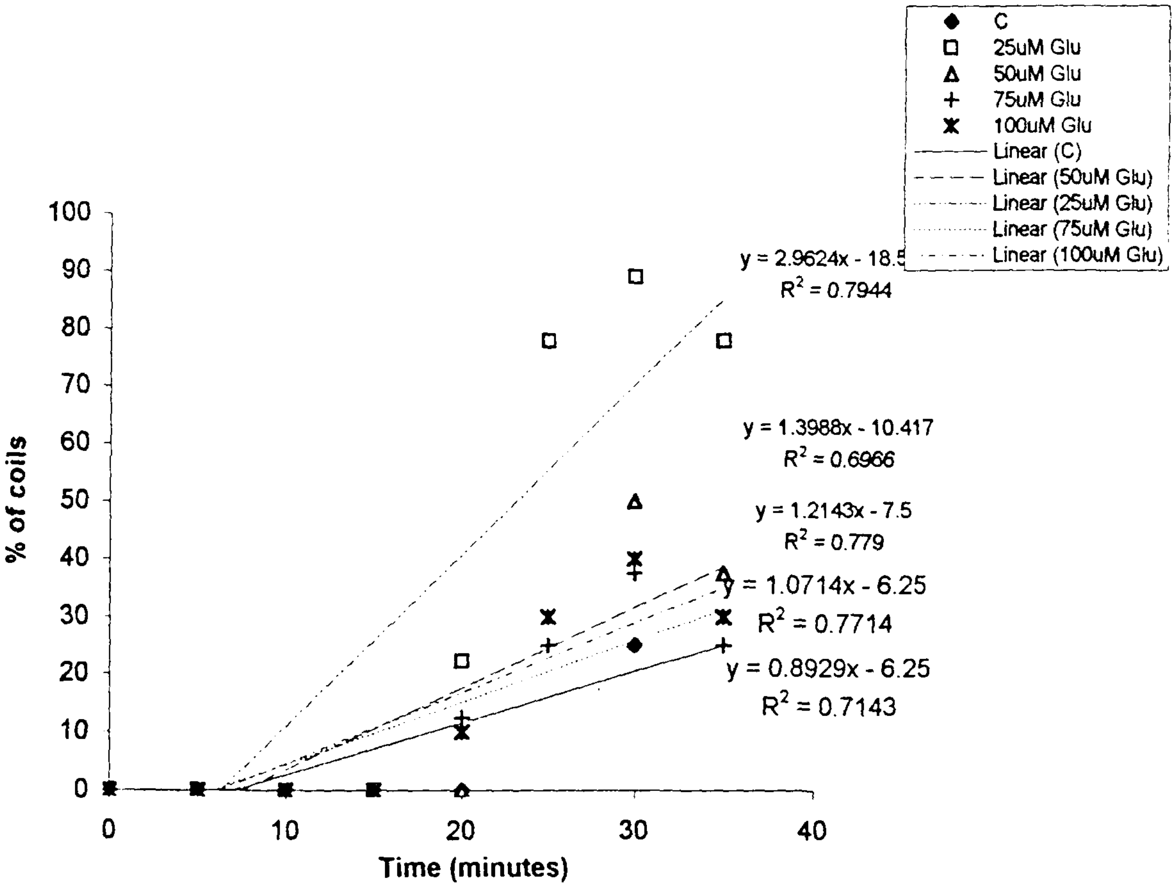


Fig. 31: Effect of glutamate on tendril coiling .

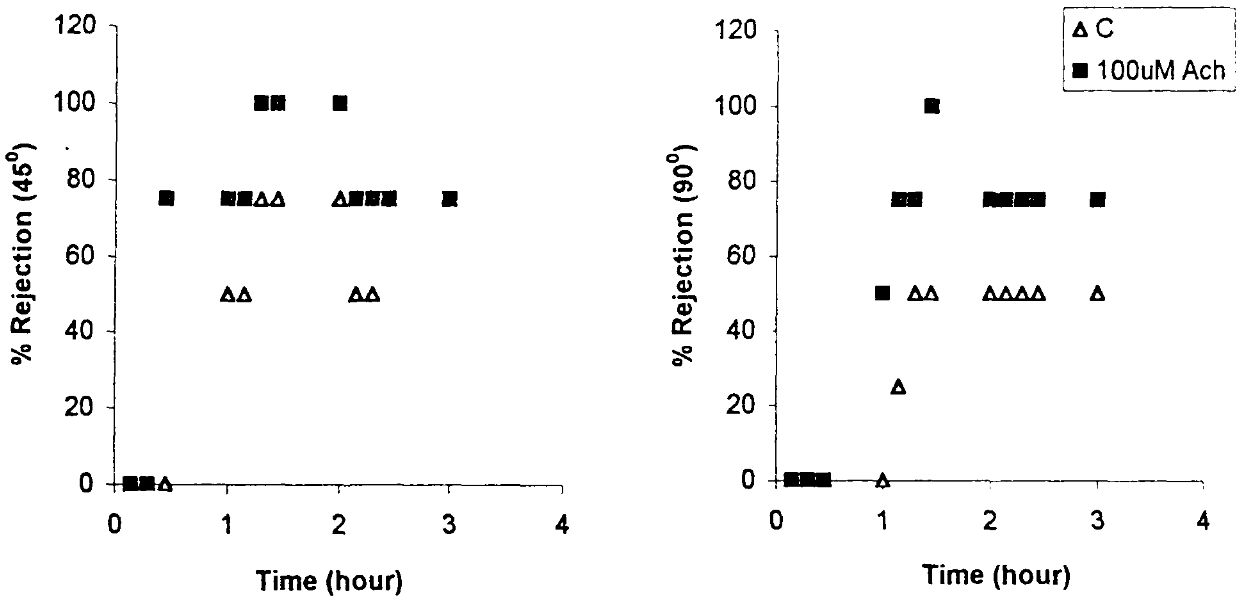


Fig. 32: Effect of acetylcholine on preference response of Cuscutta in presence of dead support.

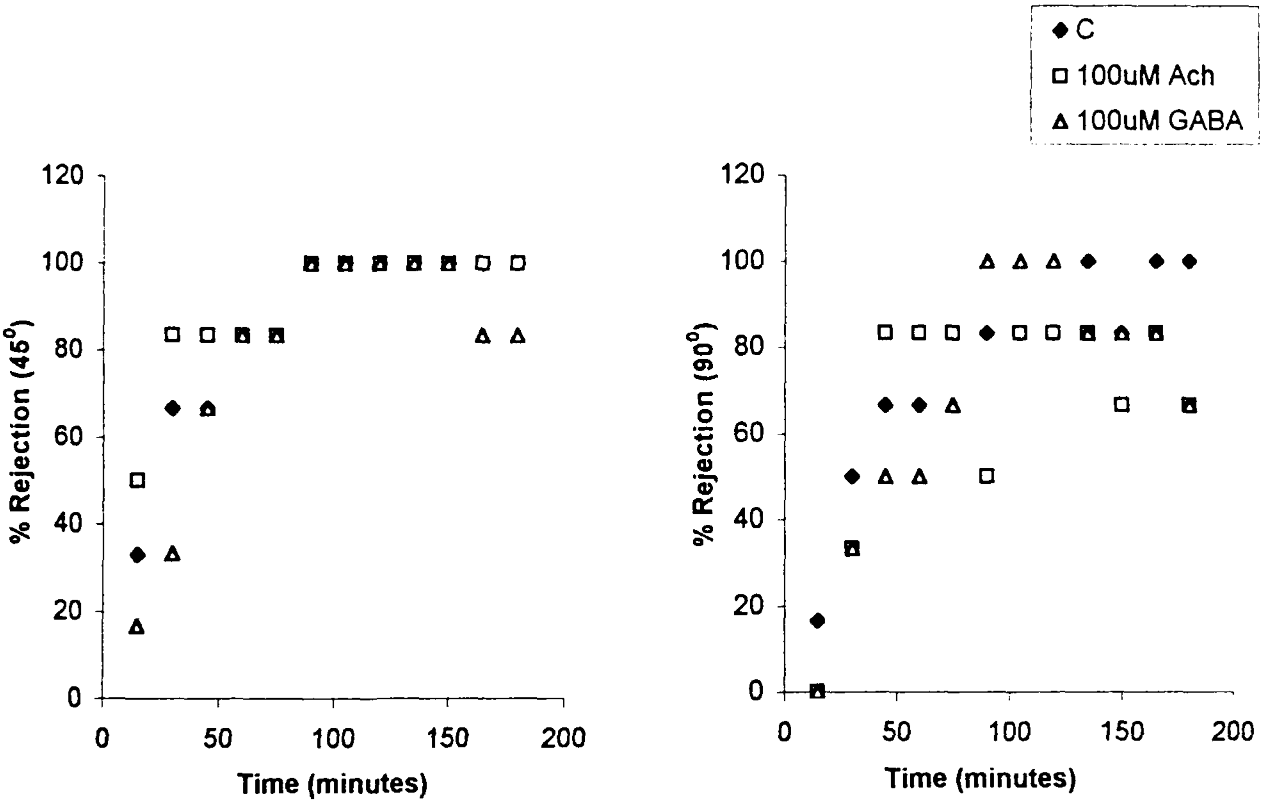


Fig. 33: Effect of GABA on preference response of Cuscutta in presence of dead support.

with 50% rejection after 60 to 75 minutes of incubation. In both the cases rejection response appeared to be slightly faster than control.

The effect of various neurotransmitters in acceptance of the live support in contrast to rejection of dead support was not clearly forthcoming. For example the effect of ACh in acceptance of live support followed the general pattern exhibited by control where there was a gradual decline with time in percentage acceptance (Fig. 34). This pattern was found to be consistent across experiments. The other neurotransmitters viz. adrenaline, glutamate and Dopa did not provide any clear pattern of acceptance compared to the control.

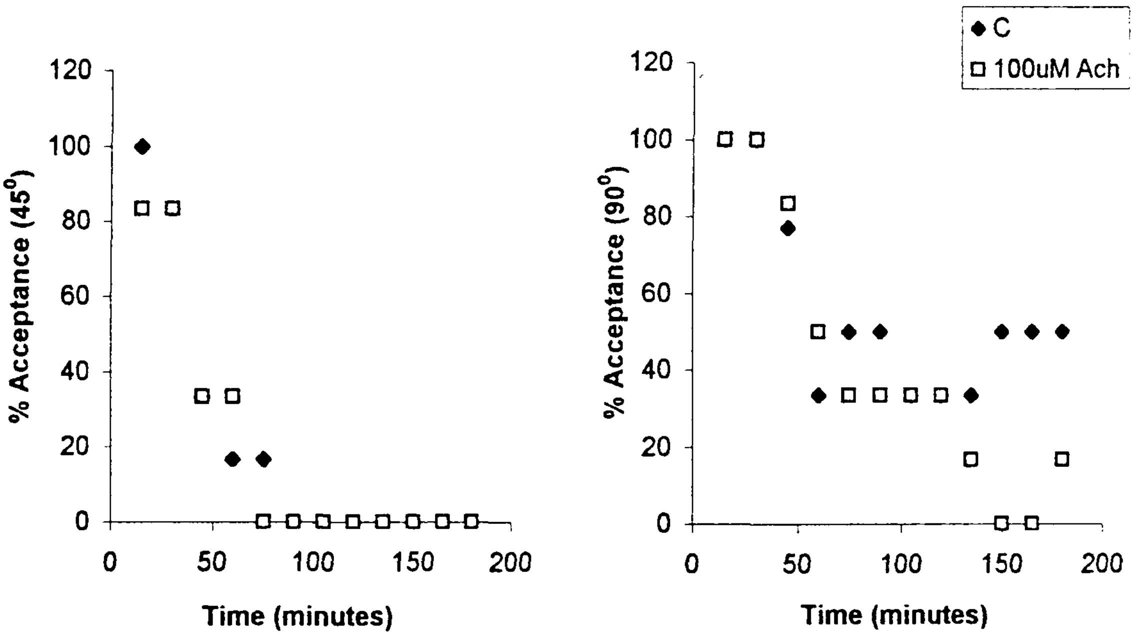


Fig. 34: Effect of acetylcholine on preference response of Cuscutta in presence of live support.

DISCUSSION

Chapter V

DISCUSSION

Neurotransmitters are one of the most important agents responsible for the cell to cell communication and macro responses in animal systems. The action of neurotransmitter is mediated through nerve cells, which interact with each other or muscles at junction called synapses. The nerve impulses or action potentials generated are communicated through the release of neurotransmitters. A number of neurotransmitters are found in animal systems and they all co-ordinate to render the necessary stimulus response functions.

Unlike animals plants don't have the nervous system, but yet are known to respond to stimuli such as heat, touch, wounding etc. It is believed that these responses are mediated by suitable signal transmission from the site of stimulus to site of action. However the underlying mechanism bringing about these responses are not clear. It is conjunctured that the responses may be mediated by ion gradient changes across cell, which are in turn modulated by hormonal changes. In fact the need for a well defined mechanism explaining the stimulus response relationship is required in plants considering the wide range of tactile motion exhibited in many species of plants.

It is in this regard that the initial discovery of neurotransmitters especially acetylcholine and the enzymes and the receptor systems associated with it in plants was very exciting and intriguing. The proposal that acetylcholine could render a similar function in plant systems was intensely debated and efforts to demonstrate its involvement in a number of physiological function was initiated. In fact it has been firmly established that acetylcholine is involved in a number of physiological processes especially those that are phytochrome mediated. It has been reported in a number of plant species and has been shown to be involved in processes such as seed germination, leaf movements, mediation of ATP levels in cells, flowering, enzyme activity, protoplast swelling, plant growth etc.

The exact mechanism underlying these varied roles of acetylcholine in plants is however not clearly understood. It is predicted that just as in animal systems, the action of neurotransmitters in plants might be based on ion gradient and might include involvement of calcium or other secondary signals such as G- proteins and cAMP.

In this study three major issues concerning the neurotransmitters in plants have been addressed. First the patterns of accumulation of neurotransmitters in plants with respect to the family of plant, habit of the plant and the particular organs or parts containing the neurotransmitter has been analysed. Following this an attempt has been made to explore the evolutionary significance of acetylcholine in plants. Second the role of acetylcholine in a few physiological processes viz. pollen growth and stomatal movement are examined. Finally the involvement of acetylcholine and other neurotransmitters in the tactile motion of plants is examined. Based on these studies it is argued that acetylcholine might be a primary recipient of stimuli in plants through which secondary cascade or messages are transferred to various sites of action. We discuss the salient results of the three major issues separately.

Patterns of accumulation or occurrence of acetylcholine in plants

Animals including human beings depend on plants for a number of curatives. A number of indigenous health systems for example Ayurveda, Siddha, Unani etc. have well documented information on the plants used to cure neural disorders in animal systems. In fact about 209 species of plant are recorded to be used in curing neural disorders with nearly 25% of them being used frequently and actively in many traditional medicinal system. Such use of plants might certainly have a basis in terms of their curative property. It is likely that many of these medicinal plants useful in treatment of neural disorders might actually control the release of neurotransmitters in human subjects or they may act at the binding site of neurotransmitter to the receptor. Either of these actions may bring about excitatory or inhibitory responses in the animal systems. Accordingly workers have tried to concentrate their efforts in unraveling the presence of neurotransmitters in such plant species and understanding the basis of their curative property.

An analysis of the plants reported in indigenous medical system having the curative property showed that most of them belong to few plant families including Cucurbitaceae, Liliaceae, Papilonaceae, Solanaceae etc. This compares well with the finding that many of these family also are known to contain acetylchoine activity. It is further interesting to note that many of these families are known for their species which exhibit rapid movements and which are tendrilous in nature. In other words the use of these plants by humans as curatives has been quite deliberate for these species of plants have evolved high levels of neurotransmitters intrinsically to probably conduct the tactile motions. However definitive conclusion on the association of these families with presence of neurotransmitters has to be weighed against their basal frequency in the native flora.

Epilepsy is one of the major disorders against which maximum number of plant families (23 out of 26 families) are used as curatives. It is likely that these plants function in suppressing the high degree of neural firing that occurs in such patients. Among the plants a large number of herbs exhibit the presence of neurotransmitter followed by shrubs, aquatic plants and trees. Among the plant parts the organ that are normally expected to receive environmental stimuli such as leaves, root tips, seeds etc. are known to contain high levels of neurotransmitter or are more frequently expected to contain neurotransmitter. Root, leaves and seed have the highest percentage of occurrence of neurotransmitter compared to other parts of the plant. If occurrence were to be random then the accumulation of acetylcholine or other neurotransmitter would have been uniformly distributed across different parts of the plant.

Leaves appear to be the major plant organ exhibiting cholinesterase activity, an enzyme responsible for the breakdown of acetylcholine to acetic acid and choline. It is likely that such high activity of cholinesterase might be evolved to forestall the herbivore damage to plants i.e. by accumulating high levels of cholinesterase the plants could reduce the levels of acetylcholine in the predators leading to a reduced activity and hence avoiding herbivore damage. Most of the neurotransmitters in the curatives derived from plants are easily extractable using an aqueous medium and thus readily adaptable in native medicinal systems. Thus we argue that the evolution of neurotransmitters like acetylcholine in plants might have significance in the primary task of signal processing or transfer and bringing about the necessary tactile motion in plants. In this process though it is still tentative it is argued that acetylcholine might in fact be the primary messenger to transfer the stimulus into the site of action. However, the detailed mechanism of these actions needs to be more intensively investigated.

Role of neurotransmitter in a few physiological processes in plants

Since the discovery of neurotransmitters and the associated enzymes and receptors in plants there has been a flood of work on unraveling the role of neurotransmitters in different physiological processes and signal transmission. For example Jaffe (1970) reported evidence for acetylcholine as a primary biochemical messenger in photoactivation of phytochrome. The involvement of acetylcholine in phytochrome mediated changes in electrical polarity of root tips (Tanda effect) is now well known. Similarly acetylcholine has been implicated in the leaf movement of plants (such as in *Mimosa pudica, Albizia julibrissin, Samanea saman*), membrane permeability to ions, flowering, seed germination, pollen germination and tube growth and in interaction with growth regulators. However the clear mechanism underlying these formative effects are not well elucidated except in a few cases. In this study the role of acetylcholine in two model systems viz. pollen germination and stomatal movement was investigated. Both of these processes have been reported to occur by ion gradients across cell membranes. For example, pollen grain germination and tube growth is reported to occur through the transfer of ions across the pollen cell membrane perhaps induced by pollen stigma interaction or the pollen media interaction. Similarly in the case of stomatal movement the regulation of guard cell movement has been well established due to the potassium-calcium flux. In both these systems there fore ion potential changes across the membranes is the driving trigger for the observed physiological processes.

These actions are similar to the action potentials generated through neurons. Acetylcholine is involved in the transmission of such signals at junction between nerves and nerves and muscles. It can be hypothesised that in plants as well, there could be a mediation of neurotransmitters in the transfer of impulses through the ion gradients. In fact, despite the lack of nervous system, cells of plants such as that of *Dionea muscipula* (Venus fly trap) and *Mimosa pudica* are known to propagate electrical stimulus as efficiently as the nerves of lower organisms (Sibioka, 1969). Such rapid movements are mediated by action potential. Thus just as bioeletric potentials are associated with ion fluxes which in turn are often induced by neurotransmitters such as acetylcholine, dopamine etc, it is proposed that in plants too, the ion-fluxes mediated physiological processes might be induced by neurotransmitters.

In this regard the results of the present study clearly showed that acetylcholine increased the pollen grain germination percentage and tube growth compared to pollen grains that were left untreated. There have been some earlier studies on the role of acetylcholine in pollen germination and tube growth. Chabra and Malik (1978) showed that acetylcholine promoted pollen tube elongation by mimicking red light. However Gharyal (un puplished) found that acetylcholine inhibited pollen tube elongation in *Lathyrus sativus*, while in few other species they don't seem to influence pollen germination.

The process of acetylcholine induced pollen grain germination could be mediated through several ways. First acetylcholine could receive a stimulus (ion flux from pollen germination media) and then generate a downstream cascade. Second acetylcholine can help transfer the ion fluxes across different cells or part of cell. For both of these the pollen grain should have necessary receptor to bind acetylcholine and render the respective actions. The studies with an antagonist of acetylcholine viz. atropine showed that the effects of acetylcholine in inducing pollen grain germination is completely reversed. In other words inhibition of acetylcholine binding to the receptor site by atropine fails to elicit germination despite all other conditions being same. These results clearly confirm the involvement of acetylcholine in the process of pollen grain germination and tube growth probably through the ion gradient changes.

The study further examined if the acetylcholine induced pollen grain germination is mediated by a down stream messenger such as calcium. In recent years a number of signal transmission mechanism has been reported by calcium calmodulin complex. It is likely that after receiving the stimulus acetylcholine transfers the ion or action potential, which leads to a second message to be taken into the cell. Pretreatment of pollen grains with calcium sequester (EGTA) or a calcium transport inhibitor such as Lanthanum again clearly inhibited acetylcholine induced pollen grain germination and tube growth. These results indicate that the acetylcholine induced increase in pollen grain germination might be mediated by the release of membrane bound calcium which then could trigger a cascade of events and result in enhancement of pollen grain germination.

Several other neurotransmitters were examined for their role in pollen grain germination such as glutamate, GABA, Dopa and adrenaline. Adrenaline and Dopa were found to have a positive effect on germination while acetylcholine, glutamate and GABA were found to have inhibitory effect.

Among the various neurotransmitters, a few bring about excitatory effect while others result in inhibitory effect. Both these however are regulated by action potentials. Under excitatory state the membrane potential changes and becomes positive and thus are able to deliver an action potential, while in case of inhibitory state membrane potential become negative and hence are unable to deliver an action potential. The observed range of actions of the various neurotransmitters seems to comply with general model of action of neurotransmitters.

Role of neurotransmitters in stomatal regulation

The role of acetylcholine and few other neurotransmitters in regulation of stomatal movement in epidermal peels of *Commelina* was investigated. Much as was obtained in pollen grain germination assay, it was found that pretreatment of epidermal peels with acetylcholine resulted in a significant inhibition of potassium influx into guard cell in presence of acetylcholine in incubation medium. The closure of stomata in presence of acetylcholine occurred in a concentration dependent manner indicating the stringent physiological role in movement of guard cell. An earlier work has also shown an inhibition of stomatal opening by acetylcholine in epidermal peels of *Vicia faba* and *Nicotiana* (Madhavan et al., 1995). The involvement of acetylcholine is further confirmed by the reversal of the action by the use of atropine, an antagonist of acetylcholine. Atropine in presence of acetylcholine enhanced stomatal opening in a dose dependent manner. In fact at 100μM atropine, the opening was restored to that of control treatment. The action of acetylcholine in stomatal regulation was also dependent on calcium wherein peels pretreated with calcium sequester showed a significant opening of the stomata.

The role of acetylcholine and other neurotransmitter in stomatal regulation at single leaf level was studied by quantifying the amount of water lost from single leaf. Though there was a reduction in the extent of water loss in acetylcholine treated leaves confirming the action of acetylcholine in controlling stomata, the decrease was not significant. Treatment of leaves with GABA resulted in reduction in rate of water loss were as dopa had no effect. These findings put together suggest that unlike hither to believed, acetylcholine and other neurotransmitters might have an important role to play in a number of physiological processes especially those mediated by ion fluxes as reported here.

Both the systems examined showed the involvement of acetylcholine and further still are shown to be calcium dependent. It would be interesting to understand the molecular mechanisms that intervene in acetylcholine induced formative effects in plants. However it would also be interesting to address the role of acetylcholine visa vis the lack of neural conducting tissue in plants i.e. how do neurotransmitters in plants operate in a nerve free system.

Role of neurotransmitter in tactile motion in plants

Plants exhibit a number of tactile motions including leaflet movement, tendril movement, stem movement in response to light, touch, electrical stimulus, chemicals or temperature. In all these movements the stimulus received as signals is most likely propagated as an action potential which then sends forth the motor responses. For example, in the touch sensitive or other rapid turgor regulated movement such as in *Mimosa pudica,* the primary stimulus changes the membrane potential in the sensing tissue. There after the signal received is propagated as action potential to the pulvinules and pulvini, which act as motor organ at the base of leaflet and leaves respectively. Phytochrome controlled rhythmic movement is also known to change membrane potential. Triyama and Jaffe (1972) examined the similarity of the process occurring in ion transport or movement in nerves and muscles and those occurring in motor cells of leaf and suggest that acetylcholine or other neurotransmitter might be involved in leaf movement of *Mimosa pudica.* Later investigations revealed the involvement of acetylcholine in leaf movements in number of plant species viz. *Albizia julibrissin, Samanea saman* and *Phaseolus multiflorus* (Satter et al., 1972). The involvement of acetylcholine has been reported in the coiling of tendrils of *Luffa cylindrica* (Hua- Baoguauy et al., 1995). They showed that plants might also have mechanism similar to the synaptic activity in nerve smooth muscle.

In this study we examined the involvement of acetylcholine in tactile motion with respect to two systems viz. tendrils of Cucurbitaceae and parasitic stem of Cuscutta was addressed. Both these systems exhibit searching behavior for the host, though in case of Cucurbitaceae it is for anchor, while in Cuscutta it is for nutrient source. The movement of tendrils has been addressed even since the time of Darwin and has formed a subject of intense investigation. In fact several scientist have viewed the movement of tendrils similar to the foraging strategy adopted by animals and hence have proposed that similar mechanisms might also underly the movement in plants.

Acetylcholine clearly encouraged the coiling of free-floating Cucurbitaceae tendril significantly more than control tendrils not treated with acetylcholine. The increased rate of coiling could be attributed to the enhanced rate of ion gradients in the tendrils leading to their movement much as an increase in action potential brought about when levels of neurotransmitters are enhanced in animal tissue. However unlike the acetylcholine mediated pollen grain germination, the acetylcholine effect was not inhibited by atropine. This suggests that perhaps the receptor site through which acetylcholine mediated tendril coiling is initiated is different from that which is involved in pollen grain germination and stomatal regulation. In other words the receptor site that might be active in acetylcholine mediated coiling could be activated by nicotinic receptor against which atropine is ineffective in blocking. Several other neurotransmitters such as glutamate and adrenaline also showed an induction in coiling though not as significant as obtained by acetylcholine.

The role of neurotransmitters in preference by Cuscutta stems using dead and live support as the host was investigated. The percentage rejection of dead support as evident by moving of Cuscutta stem away from the support showed that acetylcholine not only increased the rate of rejection but also the percent rejection among the stems pretreated with neurotransmitters. Thus for example nearly after 45 minutes of incubation, 75% of acetylcholine treated stems rejected the dead support while there was absolutely no rejection in stems kept as control. The mechanism underlying such rejection response though not clear could be hypothesised to be mediated by either stimulus from the dead support in the form of red or far- red radiation's or it could be due to certain chemical signals. In either case these stimuli need to be picked up by acetylcholine and then relayed for necessary action in terms of rejection or acceptance. In contrast to clear rejection obtained by acetylcholine when dead support were offered, clear response was not forthcoming when live support were offered. In fact the acceptance rate was very closely matched with the control. Several other neurotransmitters like GABA, dopa and glutamate also exhibited marginal increase in the rejection response of dead stems.

In summary therefore the results presented here have shown the definitive involvement of acetylcholine in few physiological processes such as pollen grain germination, stomatal regulation and tactile motion in plants confirming a few earlier studies. In this regard, the study has also shown the involvement of few other neurotransmitters such as glutamate, adrenaline, dopa and GABA on some of these signaling processes. A major feature of the study has been the demonstration that acetylcholine induced pollen grain germination and stomatal closure are mediated by a common pathway involving calcium as second messenger. It is likely that a number of signal transmission pathways in plants might be operating through the mediation of acetylcholine and other neurotransmitters. Concerted research in this regard might be useful to elucidate a number of stimulus functions in plants and to analyse the similarity in mechanism of action of the neurotransmitter between plants and animals.

SUMMARY

Chapter VI

SUMMARY

Much as in animals, in plants too, there are well recognised stimulus-response functions operating at reaction times comparable to that in animal systems. However, unlike the animal systems where the mode of such response reactions occurs through a well-developed nervous system have been well understood, in plants critical research in this area is lacking. With the discovery of acetylcholine, a major neurotransmitter, in plants, there has been efforts to understand if mechanisms similar to that in animals also exist in plants. In this study, a preliminary attempt has been made to a) examine the patterns of occurrence of neurotransmitters in general and acetylcholine in particular in plants and explore the possible evolutionary significance of these chemicals in plants and b) examine the role of neurotransmitters in a few physiological functions in plants and to understand the underlying mechanisms of their action.

Patterns of occurrence of neurotransmitters in plants: About 209 plant species are recorded to be used in the treatment of neural disorders in many traditional health care systems. It is likely that many of these plant species indeed contain neurotransmitters, which help in alleviating the neural disorders. The present study indicated that the occurrence of neurotransmitters is largely concentrated in plant species belonging to a few families such as Cucurbitaceae, Liliaceae, Papilionaceae and Solanaceae; these families incidentally also constitute the major component of plants used for curing neural disorders. It is interesting to note that many of these families are known for their species which exhibit rapid movements and which are tendrilous in nature. Among the plants, herbs are more frequently reported to contain neurotransmitters followed by shrubs, aquatic plants and trees. Further, among the various plant organs, leaves, root tips and seeds seem to contain more frequently high levels of neurotransmitters than are other parts of the plant. The study suggests that there might be a non-random distribution of neurotransmitters in the plants and particularly in those that exhibit tactile motions. However definitive evidence of such association has to be weighed against the basal frequency of the plant species in the native flora. It is argued that the evolution of the neurotransmitters in plants might have been primarily designed to aid in signal transmission to bring about the varied stimulus-response functions.

Role of neurotransmitters in plants: The role of neurotransmitters in a few physiological processes such as pollen grain germination and tube growth and in the regulation of stomatal opening and closing was investigated. The study showed that a) acetylcholine significantly increased the in vitro germination and tube growth of Sunflower pollen grains and b) acetylcholine application to epidermal peels of Commelina, resulted in the significant closure of the stomata. Both of these processes were found to be reversed by atropine, an antagonist of Acetylcholine receptor site. These results suggest the involvement of the neurotransmitter in the process of pollen grain germination and in the regulation of stomatal opening/closing. The study further showed that the underlying mechanism of such action could be mediated by the involvement of the divalent cation, calcium. Pre-treatment of pollen grains and or epidermal peels with calcium sequester or transport blockers completely reversed the effects of acetylcholine. It is hypothesised while acetlycholine might serve as the stimulus receiving moeity in the plants, the manifestation of the physiological processes might depend upon the downward cascade effected by the release of the second messenger, calcium from the membrane surface.

The study also examined the role of acetylcholine in conducting the tactile motions in plants in two model systems, namely, the coiling behaviour of tendrils in Cucurbitaceae plants and the host-searching behaviour of the parasitic stems of Cuscutta. The results suggest that acetylcholine might be involved in both of these tactile motions as well in plants. Tendrils treated with acetylcholine tended to coil faster while Cuscutta stems treated with acetlycholine tended to reject dead supports faster.

The work presented here is a preliminary attempt to understand the role of neurotransmitters in plants and has perhaps raised more questions than answered. It has always been enigmatic as to why plants produce the score of chemicals that are used in the apparent cure of a number of mammalian disorders. The case of curative against neural disorders is one such extreme example. Systematic studies show that perhaps these "curatives" are mere spin-offs, of a more elaborate evolutionary plan that have occurred in plants. The results of the present study seem to confirm this view and hope to catalyse further research in this area.

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Chapter VII

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