The Biophysical Mechanisms of Electrical Stimulation on *in-vitro* Growth and Differentiation in *Populus deltoides* and *Nicotiana tabacum*

THESIS

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By
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Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

-A. Szent-Gyorgyi

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CERTIFICATE

This is to certify that the thesis entitled "The Biophysical Mechanisms of Electrical Stimulation on *in vitro* Growth and Differentiation in *Populus deltoides* and *Nicotiana tabacum*" submitted by RAJIV DUTTA, ID No. 1994PHXF010, for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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Contents

Abbreviations Used in Thesis

+ve	explant in positive polarity
-ve	explant in negative polarity
IAA	indole acetic acid
2,4-D	2,4-diphenoxy acetic acid
BAP	6-benzylaminopurine
AdS	Adenine sulphate
TIBA	tri-iodobenzoic acid

The electrical pattern is intimately related to the morphogenetic processes and polar or vectors properties of cell and tissue functions. One function of this electric field or pattern to act as a "directive force" in laying down new structures.

-Elmer Lund (1947)

Chapter 1

Introduction

It is well established that all biological systems contain dipolar components capable of electric oscillations. The interaction between the giant dipoles comprising a biological system through long range Coulombs forces will established a branch or several branches of longitudinal electric modes in a frequency of 10^{11}- 10^{12} Hz. The oscillations involve regions of biological membrane or substantial segments of macromolecules such as proteins, DNA, etc. (Wu, 1990). If sufficiently large energy (electric current) is locally supplied to one component of the biological system, it will be shared by all other components and will become channeled into lowest energy mode which represent Bose-Einstein condensation (characteristic low temperature effect applicable to helium and superfluidity) without temperature restriction. This phenomenon serves not only as mechanism of energy storage but also as regulator/ stimulator for specific bioprocesses such as cell division and protein synthesis (Vaidyanathan, 1990; Frohlich, 1970). Information on the effect of externally applied electric current on biological growth originates from the studies on the polarized directional growth of biological systems, especially abnormal tissues. Our knowledge on the regulation of directional growth by external electric current in unpolarized multidirectional growing tissue, especially of plants, is scanty. To understand the present state-of-art, aims and objective of the present investigation, it is essential to dwell upon the earlier investigations made elsewhere.

1.1 Historical Background

The earliest report on the intimate relationship between electricity and life dates back 1977 by Fraze. Bose (1907) first reported regulation of physiological process by electric phenomenon in plant tissue in *Mimosa pudica*. Following the report of Bose (1907), much has been written on the intimate relationship between electricity and life. It is now well known that a large variety of both animal and plant tissues are associated with and affected by electric fields.

1.1.1 Electric Fields in Plants

Living cells contain aqueous phases, which are separated from each other and form the external aqueous phases by membranes through which exchange of ions and molecules take place. The interior of the cell is maintained at a different ionic composition from that of the extra cellular environment (in most plant cells at much higher concentration) by ionic or molecular pumps, requiring a continual expenditure of metabolic energy (Jacobson and Saier, 1989). Ionic diffusion through a membrane establish an electric field which keeps the total flux of positive and negative ions through the membrane at Donnan's equilibrium (as determined by concentration differences and electric potential differences across the membrane, ion pumps, and possibly bulk water movement). The interior of the cell is always more negative in potential than the external solution by 70 to 150 mV. According to Courad (1990), the cell membrane exhibit pairing

interaction between proton and electron which is dual to the conventional metallic superconductivity occurring at a higher temperature in biological systems.

Most of the recent investigations of plant electricity have been carried out at the cellular level. Attempts have been made to examine in detail the interrelationships among the electrical properties of cellular membranes, the potential differences and ion concentration differences across them, and the fluxes of ions through them. Because of the difficulties of working with cells of normal size, most investigators have used unusually large cenocytic cells of certain fresh water and marine algae. The aim of these studies is to determine which ions cross a membrane passively (their movement being determined by concentration differences and electric potential differences alone), and which ions are pumped actively.

Very good reviews on the electrochemical theory of ion movement in plant cells have been made by Briggs et al. (1961), Dainty (1962) and Dainty and Ginzburg (1964).

Comparatively few reports are available on the electrical properties of individual cells of higher plants and their relationship with ion distribution. Etherton and Higinbotham (1960) in their studies on the electric measurement using fine

microelectrodes, observed potentials inside the cells about 100 mV more negative than the KCl bathing medium in cells of various plant tissues. For *Avena* root hair cells, the major part of the potential appeared to be across the plasmalemma, as in case of large algae. In all tissue examined, the vacuolar potential appeared to be almost independent of the ionic concentration of the bathing medium after exposure to it for periods of 10 to 30 hours (Etherton and Higinbotham, 1963). Higinbotham et al (1964) in short term experiments with cells of the *Avena* coleoptile, found that an increase in the external concentration of K^+ from 1 to 10 mM caused the membrane potential to fall by 39 mV with the corresponding fall for Na^+ of about 5 mV. It was concluded that the membrane potential is actually the diffusion potential dominated by K^+. It was also observed that the effect of changes in magnesium or anion concentration appeared to be small, but an increase in Ca^{++} or H^+ caused the potential to rise, indicating their effect on active transport mechanism (Higinbotham et al., 1964 a and b)

Higinbotham et al. (1964 a and b) and later Busa (1986) measured the electrical resistance of cell membranes in the *Avena* coleoptile by inserting two probes into the cell, one of the probes was used for the passage of current and the other for measuring the resulting membrane potential change. They obtained a mean value of 1300-ohm cm^2, which increased with the decrease in external K^+ concentration or increase in the Ca^{++} concentration. They concluded that passive fluxes of K^+ largely account for the measured resistance, increased Ca^{++} apparently reduce the membrane permeability to K^+.

Sibaoka (1962) in his investigation on membrane potentials in the excitable tissue of *Mimosa*, found that elongated parenchyma cells in the protoxylem and phloem of the petiole had a membrane potentials of 150 mV (interior negative) in weak salt solutions but other cells in petiole sections had smaller resting potentials. Only the parenchyma cells were found to be excitable with action potential spike heights at about 130 mV. The transmission of the electrical wave takes place readily from cell to cell apparently through electrical conducting paths and a number of cells have to be stimulated simultaneously before the wave travels to the petiole (Siboka, 1960). The wave transmission is of the order of 2 to 3 cm per second, which can be raised significantly by immersing the petiole in NaCl solution. Similar reports have also been made in *Dionaea* (Di Palma et al., 1961) and *Biophilum* (Guhathakurta and Dutt, 1963; McGillivary and Gow, 1987; Takeuchi et al., 1988). D'Inzeo et al. (1993) demonstrated that the sensitivity of electrical channel response depends on the frequency and amplitude of electromagnetic stimulation.

1.1.2 The Role of Bioelectric Fields in Morphogenesis

The speculations are whether the electric fields set up in and around developing tissue are merely by-products of the development or whether they themselves take part in controlling further development through the electrophoretic movement of auxins (Graham, 1964). Many attempts have been made to modify the rate of

plant growth by either the application of electrostatic fields or the passage of electric current through the tissue (Briggs et al. 1926). Most of these treatments have either had no effect or caused reduced growth and sometimes tissue damage. Murr (1963) found that tips or blades of grass were damaged in strong electrostatic fields up to 2000 volts. cm^{-1}. According to him the effect may not be electrostatic but may be due to lethal current densities caused by corona discharge from cells near the tip.

Schrank (1959) and Umrath (1959) have demonstrated through various experiments that the *Avena* coleoptile can be induced to bend through application of current of the order of 10 mA. For few minutes these effects are related to the availability of auxin since auxin-depleted coleoptiles do not bend. It is perhaps not surprising that attempts to modify the delecate bioelectric pattern by the passage of bulk currents from external electrodes did not clearly show whether or not the plants development is moderated by its own electric field. The electric current or the field constitutes a "directive force for morphogenisis" of the hyphal tip.

1.1.3 The Growth Current

The research on endogenous electric currents began when Jaffe and Nuccitelli (1974) invented the vibrating electrode instrument capable of measuring the minuscule electric field generated by single cells in the surrounding medium. The

probe is exquisitely sensitive, measuring potential differences in the nanovolt range and by moving the electrode around the cell one can generate a spatial map of current distribution. Much of the tedium associated with this procedure has been eliminated in modern version of the vibrating probe, which determine current vectors in two dimensions automatically.

By the use of the vibrating probe, Jaffe and Nuccitelli (1974) mapped currents in space as well as in time around diverse cells and small organisms viz. eggs and embryos, shoots and roots, muscles and nerves, algae, amebas, fungal hyphae, and filaments of cynobacteria. The results establish that transcellular electric currents are widespread and possibly ubiquitous among eukaryotes. The current patterns are visibly correlated with anatomy and function and often with subsequent development or differentiation (Jaffe, 1979, 1981; Jaffe and Nuccitelli, 1977; Weisenseel and Kircherer, 1981; Nuccitelli, 1983, 1986, 1988; DeLoof, 1986).

1.1.4 Electric fields of Bulk Plant Tissues

The source of the electric field around plant organs and tissues has been the subject of much speculation (Lund 1947). Since the cellular membrane potential is a function of external ionic concentration, the surface of cell would no longer be equipotential in the presence of a concentration gradient in the bathing medium. Ionic concentration gradients in the intercellular medium of developing tissue generates minute electric fields around individual cells, which in turn frequently add together to give a macroscopic field of observable magnitude. The

field would cause the flow of electric current in and around the tissue immersed in a conducting medium and the ohmically dissipating energy being supplied by ionic gradient across the membrane.

Hydrogen ions arising from the synthesis of organic acids in developing tissue may also contribute subtantially to the bioelectric field (Fensom, 1959). The cell wall in *Chara* contains polyuronic acids at concentrations approaching 1 $eq.kg^{-1}$ (Dainty et al., 1960). It is reasonable to assume similar high concentration of fixed anion exist in the walls of higher plant cells. In comparison to the sap of large algae, the sap of higher plant cells is rich in organic acids which are possibly held there by membranes even slightly permeable to them. In many plants, the total organic acids content are about 60 $meq.litre^{-1}$ in sap of root cells and about 160 $meq.liter^{-1}$ in storage tissue (Poole and Poel, 1965).

The hydrogen ions produced during the synthesis of acids diffuse away from the production site along their concentration gradient. These H^+ are replaced in the tissue by cation such as K^+, Na^+, and especially by Ca^{++} in case of the cell wall. Since the hydrogen ion has greater permeability than the other cation across the membranes, a proton gradient would occur across membranes making the transport site more negative. No electric current would be observed if the replacement cations were to enter the root through exactly the same path by which the H^+ leaves. If these paths do not coincide, a bulk electric current would be observed flowing around closed pathways in and around the tissue.

Scott and Martin (1962) reported total currents of about 0.2 mA flowing in weak KCl solution surrounding *Vicia faba* roots due to a small difference between the path of entry of K^+ and exit of H^+. They observed that the patterns of current flow were similar around roots grown in solutions containing 10^{-3} eq.liter^{-1} of K^+, Li^+, Mg^{++} or Ca^{++}, and the solution adjacent the meristematic region was more negative than that near other parts of root. With Na^+ on the other hand, the most negative region occurred where the root was elongating most rapidly. A change of anion had no effect. By using radioisotopes, these authors found significant difference in the paths of entry of Na^{22} and K^{42} into the root and the influx was greatest in the region where the external solution was most negative. This is to be expected if these ions carry the in-flowing electric current across the plant surface. The electric source in the root was shown to generate about $4x10^{-9}$ watts of power, which was a very small fraction (approximately 10^{-5}) of the rate of generation of respiratory energy in the root.

Scott (1957) found that some *Vicia* roots in weak salt solutions spontaneously generated electric oscillations which were closely sinusoidal with periods ranging from 5 min (i.e. frequency of 12 hr^{-1}) at 25 °C to 20 min (i.e. 3 hr^{-1}) at 10 °C (Gunther and Scott, 1966). The oscillations in the electric field, normally detected in the external solution, could also be demonstrated with microelectrodes within individual cells (Jenkinson, 1962). Scott (1957) proposed that a feedback control pathway in these roots become over corrected causing it to hunt. It has

been suggested that the bioelectric current through the tissue influenced the distribution of auxin to sites within the tissue where it affected membrane permeability. Changes in permeability altered the membrane potential thereby the bulk current flow

Experiments showed that the root with steady electric field could be forced into an oscillatory state by subjecting it to rhythmic changes in the osmotic pressure, auxin concentration (Jenkinson and Scott, 1961) and temperature (Gunter and Scott, 1966) of the bathing medium. The electric response agreed with that expected for the feedback model being highest for forcing oscillations having periods close to that of the spontaneous oscillations, and lagging or leading in phase in the predicted manner. Swamping of root with strong solutions of indole acetic acid (IAA) or the auxin antagonist 2,6-dichlorophenoxyacetic acid inhibited the oscillation behavior (Jeckinson, 1962). The observation (Gunther and Scott, 1966) that the frequency of both natural and resonant oscillations varied linearly with temperature over the range 10 °C to 27 °C suggested that the feedback loop included oscillations that were not purely thermochemical.

Newman (1963) and Robertson and Astumian (1992) also observed the oscillatory bioelectric behavior in *Avena* coleoptile. They observed that a change in the downward flux of auxin in the transport stream was accompanied by an oscillatory distribution of electric field at the surface of the coleoptile. The effect could be induced by unilateral illumination of the coleoptile or by the application

of IAA to a decapitated coleoptile. When electrical contact with points on the coleoptile was made through continuously flowing streams of weak salt solution, the electric disturbance traveled down the coleoptile at a rate similar to that of auxin transport thus confirming the close association between the two (Newman, 1963).

Graham and Hertz (1962, 1964) used a vibrating electrode to sense the potential of a plant surface close to it without being in mechanical contact. They observed that when a *Zea mays* coleoptile was placed horizontally to its axis, it developed a lateral potential difference of about 80 mV (the lower surface being positive) after a latency period of about 20 min. According to Grahm (1964) the asymmetric application of IAA to decapitate coleoptile produced lateral potentials similar to those produced by gravity and the side supplied with auxin being positive. The decapitated coleoptiles looses their ability to produce the geoelectric response progressively form the cut end down to the coleoptile tip approximately at 15 mm. hr^{-1}(i.e. the speed of auxin transport) but the geoelectric response could again be restored when the cut end resumed auxin production. Johnson (1965) has used the vibrating probe to observe lateral potential differences in unilaterally illuminated coleoptile. He observed that the illuminated side became about 50 mV negative as compared to the dark side with a latency time of about 30-min. All these observations support the conclusion that the geoelectric effect is associated with the asymmetric distribution of auxin in the horizontal coleoptile with a higher concentration on the lower side. Grahm (1964) believes that the delay in

generation of electric field is an indication that it is an effect of auxin redistribution and not a cause of it.

Robinson and Cone (1980) germinated *Pelvetia* zygote in gradients of calcium ionphore A23187 and found that the rhizoids predominantly formed on the higher side of the gradient, presumably in the region of maximal calcium influx. Brownlee and Wood (1986) and Brownlee and Pulsford (1986) reported a pronounced maximum of free calcium ions at the rhizoid tip itself. Speksunder et al. (1989) demonstrated that injection of BAPTA type calcium buffers inhibit self-polarization of *Pelvetia* zygotes possibly by dissipating a local calcium gradient in the immediate vicinity of the rhizoid tip.

The vibrating probe monitors only the flow of charge but cannot, by itself, ascertain whether an inward current represents the influx of cations, the efflux of anions, or both. Dissection of the current is usually done with experiments in which a particular ionic species is omitted. For example, if potassium ions carried that inward current, omission of K^+ from the medium should abolish the current. Infact, omission of K^+ might diminish a sodium current by inhibiting the Na^+, K^+-ATPase, or diminish a proton current by limiting the net exchange of protons for K^+. The relation between Ca^{++} uptake and transcellular current is difficult to assess as complete removal of extracellular Ca^{++} is deleterious to the permeability and stability of the plasma membrane. The intertwined ion movements across the

plasma membrane of diverse cells suggests numerous potential pitfalls, not only for ion substitution but for the inhibitors and mutants as well (Harold, 1986).

1.1.5 The Electrical Properties of Plant Membranes

Several important investigations have recently been made on the electrical properties of the membranes of large algal cells. Using microelectrodes inserted into the vacuole and cytoplasm of *Nitella*, Walker (1960) demonstrated that the electrical resistance of the outer boundary of the cytoplasm (the plasmalemma) was much greater than of the inner boundary (the tonoplast). He also found that the major part of the potential difference between vacuole and external solution occured at the plasmalemma. In *Chara australis*, Findley and Hope (1964) reported typical resistance values of 12.1 kohm. cm^2 for plasmalemma and 1.1 kohm. cm^2 for tonoplast with the corresponding membrane potentials of −150mV and -11 mV, and the cytoplasm being most negative. Studies on K^{42} and Na^{22} flux measurement in fresh water algae *Nitella* (MacRobbie, 1962) and *Chara* (Hope, 1963) confirmed that the plasmalemma act as the main barrier to ion diffusion. In the marine alga *Halicystis ovalis*, the main potential difference also occurs at the outer membrane (Blount and Levedahl, 1960) but in *Valonia ventricosa* the tonoplast potential is larger than that of plasmalemma (Gutknecht, 1966). Hope and Walker (1961) in their studies on *Chara*, showed that the membrane resistance and potential were mainly dependent on the concentrations of ions (particularly K^+) in the external medium. Both the resistance and the

magnitude of the potential decrease with increase in the concentration of K^+. In these experiments, transient effects during equilibration with the Donnan phase of the cell wall were avoided by changing K^+ concentration at the expense of another cation, Na^+. The membrane being nonohmic, had greater resistance in hyperpolarized than depolarized condition. Their results fitted reasonably well in the model based on the Goldman (1943) assumption that the electric field strength in the membrane is uniform and the membrane is about ten times more permeable to potassium than the sodium.

Williams et al. (1964) demonstrated that the measured membrane resistance in *Nitella translucens* is only about one twelfth of the value predicted from flux measurements assuming that ions cross the membrane independently. According to these workers, this discrepancy can be accounted for by assuming that the ions do not pass independently through the membrane but move in single file through pores containing about 12 ions. An alternative explanation by these authors is that the passage of an electric current through the membrane during resistance measurement is accompanied by an electrosmotic water flow in the direction of cation movement (Fensom and Dainty, 1963) that in turn increases the cation flux and reduces the measured electrical resistance.

An important new development in membrane theory has emerged from consideration of the properties of a boundary between layers containing fixed charges of opposite sign. Mauro (1962) pointed out that a region on either side of

such a boundary has low concentration of mobile ions (the depletion layer) and will therefore act as a barrier to ion diffusion. Furthermore, the boundary exhibits 1 to 2 $\mu F.cm^{-2}$ rectification and capacitance, which is comparable with that of plant membranes (Findlay and Hope, 1964; Oda, 1976; Williams et al., 1964). These properties are similar to those of a solid state *pn*-junction where the charge carried through holes and electrons rather than positive and negative ions. Coster (1965) has reported that there is good quantitative agreement between the voltage current characteristics of the membranes of *Chara* and *Nitella* and those predicted for the fixed charge lattice model. The model particularly accounts for the nondestructive punch-through property of the plant membrane hyperpolarized by about 200 mV. The membrane current increases to a very high value at this potential. Using plausible values for the concentration of fixed ions in the membrane, Coster (1965) calculated a membrane thickness of 7 mμ, which is close to the accepted value. George and Simons (1966) point out that some of the simplified assumptions could lead to significant errors in quantitative estimates of membrane properties. In order to account for observed membrane resistance, these workers concluded that either ionic mobility in the membrane should be about one-thousandth of their free space value or the ions could only pass through pores in the membrane which occupy this fraction of the membrane area. The fixed charge lattice model of membrane structure has many attractive features and needs more extensive study.

The development of a technique for replacing the sap of *Nitella* cells with artificial solutions without apparently affecting the health of the cells (Tazawa, 1964) has great value in investigations of membrane properties. Tazawa and Kishimoto (1964) reported that replacement of K^+ in cell sap with Li^+, Na^+, or Rb^+ had little effect on the potential of the vacuole, although this was apparently before the cytoplasm had equilibrated with it. Kishimoto (1965) has examined the effect of concentration of KCl on the membrane potential and resistance in both sap and external medium, in experiments having the sap tonocity maintained at normal levels with mannitol at reduced salt concentration. The potential difference between vacuole and external solution decreases to lowest, as the internal ionic concentration became equal to the external concentration. This was attributed to the cell wall or to an effect of Ca^{++}, a necessary constituent of the artificial sap. Alternatively, the nonzero potential was caused by an electrogenic component in one of the pumps. The tonoplast potential became higher at low vacuolar concentrations, possibly due to a Donnan effect of indiffusible anions in the cytoplasm. Changes in KCl concentration in either external medium or vacuole had a marked influence on the total resistance between these phases, but the resistance remained same for the same pair of concentrations irrespective of their side of the membrane.

1.1.6 Action Potential across Plant Membranes

When the membrane of *Nitella* or *Chara* is depolarized below a threshold value, a characteristic electrical response known as an action potential is generated. If the cell is depolarized locally, the action process spreads over its entire surface as a wave at a speed of few centimeters per second depending on the external medium but seldom transmitted to an adjoining cell (Sibaoka, 1966). Findley and Hope (1964) have reported that the potential changes of the vacuole of *Chara* during the action process involve a rapid fall of about 25 mV in the potential across the plasmalemma with a recovery period of about 3 seconds, followed by a slow increase in potential across the tonoplast to about 50 mV with gradual recovery to the resting level after about 20 seconds.

The resistance of the tonoplast drops by a factor of about 4 during the action period (Findlay, 1964). Findlay and Hope (1964) concluded that the lowered electrical resistance of the plasmalemma is due to a large transitory increase in its anion permeability and the resulting cytoplasmic efflux of Cl^- caused the membrane depolarization. According to these workers subsequent recovery of the potential to the resting level was due to loss of cations (particularly K^+) by the cell. The observation that the action process was very sensitive to Ca^{++} concentration led them to suggest that the inward movement of Ca^{++} caused the initial membrane depolarization (Findlay, 1961; Hope, 1961). Mullins (1962) using *Nitella* cells loaded with Cl^{36} found an increased efflux of Cl^- accompany the action process. Hope and Findlay (1964) confirmed these observation in

Chara. Kishimoto and Ohkara (1966) reported that the loss of ions due to the action process upsets the osmotic balance of the cell with associated water loss. With the exception of *Valonia* (Gutknecht, 1966) all cells examined have an inwardly directed anion pump which caused Cl^- accumulation.

Spanswick and Williams (1964) located the anionic pump at the plasmalemma although many reports indicate that part of it is at the tonoplast (Coster, 1966; Kishimoto and Tazawa, 1965). It is well known that K^+ moves either passively or actively inwards and Na^+ is actively extruded. This is particularly true of the marine algae (Blount and Levedhal, 1960; Gutknecht, 1966). Reports of very low Na^+ concentrations in the flowing cytoplasm of *Nitella* appear to indicate that sodium pumps occur both at the plasmslemma and the tonoplast, each directed away from the cytoplasm (Kishimoto and Tazawa, 1965; Spanswick and Williams, 1964). Ca^{++} is also far below its equilibrium concentration inside the cells, possibly because of its low permeability through the plasmalemma. Spanswick and Williams (1964) suspected that the active inward transport of calcium at the tonoplast regulates the polarization of *Fucoid* embryos. Kropf and Quatarno (1987) concluded that calcium influx into the rhizoid tip is required for its extension, presumably because Ca^{++} ions controlled the exocytosis of precursor vesicles.

It has been proposed that the flow of electric charge through the hyphae is due to the partial segregation of proton pumps from proton-coupled transport systems

(Kropf et al., 1984; Gow et al., 1984). The protons expelled from the cytoplasm by the H^+-ATPase, flow back across the plasma membrane, down their electrochemical gradient. Since the porters are preferentially localized in the apical region, a longitudinal current of protons is established through the apical region (Schreurs and Harold, 1988; Harold, 1986, Harold and Harold, 1986). Potapova et al. (1988) inferred that electric current enters the apical region to gives rise an electric field across the cytoplasm. By this mechanism, *Achyla* generate a substantial electric field across the apical cytoplasm - as steep as 0.2 V/Cm. (tip positive). It is now more and less established that under certain conditions hyphae extends normally while electric current 'flows out' of the apical region (Thiel et al., 1988; Kropf et al., 1984; Brawley and Robinson, 1985).

1.1.7 Currents and Calcium in Pollen Tube Extension

The germinating lily pollen grains form a classical system for the study of transcellular ion currents (Robinson, 1985). Pollen grains were germinated on buffered salt solution solidified with agar and supplemented with calcium and potassium ions. Electric currents begin to circulate through the grain within an hour of sowing. In *Pelvetia*, the locus of maximal inward current formed the site from where the germ-tube emerged several hours later. Once the outgrowth began, the pattern of electric current became clearly defined: current of 0.1 to $\mu A/cm^2$ flowed into whole of the anterior segment of the germ tube, exits from the grains and the basal part of the germ tube (Weisenseel et al. 1975). The

current indicates the net uptake of potassium ions into the tube in exchange of protons from the grain (Weisenseel and Jaffe, 1976).

The requirement of calcium ions for the extension of tube is well established. Jaffe et al. (1975) showed by low temperature autoradiography that calcium ions accumulate in the tip of germ tube. Subsequent reports confirmed that germ tubes maintain a steady state calcium gradient, which is maximum at the apex. The existance of a calcium gradient, sequestered in membrane vesicles, have been detected and determined with help of cholorotetracycline (Reiss and Herth, 1979; Reiss et al., 1985) and the scanning proton microprobe (Reiss et al., 1985) techniques. Observations with quin-2 suggest that there may also exist a parallel gradient of free cytosolic calcium (Nobiling and Reiss, 1987). It is well-established that the compounds blocking calcium channels in the plasma membrane inhibit tube extention (Reiss and Herth, 1985; Picton and Steer, 1985; Weisenseel and Kircherer, 1981; Helper and Wayne, 1985). According to Robinson (1985) no steep gradient is demonstrable in normal pollen tubes but only damaged ones show substantial elevation of free Ca^{++} in their apical region.

According to Picton and Steer (1982), calcium influx into the extreme tip raises the local cytosolic free calcium concentration and favours the exocytosis of precursor vesicles for the production of new wall and membrane. In the region just behind the tip, membrane vesicles maintain a low steady-state level of free calcium ions. This is a prerequisite to the extension of apical action meshwork.

(Reiss and Herth, 1979, 1985; Picton and Steer, 1983, 1985). On the contrary Kauss (1987) suggests that the elevated level of apical calcium may be related to the proportionate deposition of callose and cellulose at the tip.

1.1.8 Biological Effects of Applied Electric Fields

There has been scattered reports that small electric fields can impose their own polarity upon cellular growth, development, or behavior. Lund (1947) lists diverse examples and recounts his findings that the dissociated cells of *Polyp obelia* reassociate in the presence of an electric field, so that stems grow towards the negative pole but buds face the positive pole. Jaffe and Nuccitelli (1977) gave several examples of cellular polarity under electric current from both animal and plant kingdoms. Several types of cultured animal cells when subjected to electric fields as small as 1 to 10 mV across the cell width, migrate preferentially toward the cathode (negative pole). Electric fields of this magnitude not only orient the extension of neurites and fungal hyphae, but also the locus of outgrowth from zygotes, spores, pollen grains, and protoplasts. Electric fields also stimulates the motility of animal cells, and the frequency of neurites sprouting and hyphae branching (Lund, 1947; Nuccitteli, 1978). It is reasonable to presume that applied electric fields may modulate or override endogenous electric changes. These reports stimulate interest in researches on the role and effects of applied electric fields on endogenous electric fields for the localized growth and development in plants.

Jaffe and Nuccitelli (1977) pointed out that, the morphology and physiology of the cells appear to be quite normal under the minimal electric field. According to these workers applied electric fields do not alter the patterns of cellular organization but only provide a kind of sensory cue. Animal cells consistently orient themselves toward the cathode, but walled cells likely favors the anode. In *Pelvetia*, the polarity or rhizoid outgrowth was found to vary with the batch of eggs. Of 16 batches, 11 germinated on the positive pole side, two on the negative pole side, and in three polarities were a function of the field strength (Peng and Jaffe 1976). Among the fungi, germination of *Neurospora* conidia was oriented toward the positive pole, as also the extension and branching of its hyphae. But *Aspergillus* hyphae grew and branched toward negative pole and the polarity of its germination remained unaffected by the electric field. In *Trichoderma*, hyphae grew toward the negative pole but branched on the side facing the positive one. Yet in all these organisms, electric current flowed into the apical region (McGillivray and Gow, 1986, 1987).

Jaffe and Nuccitelli (1977) proposed possible mechanisms by which applied fields may exert biological effects. According to them the resistivity of the plasma membrane is much higher than those of medium and cytoplasm if spherical cells are suspended in liquid and subjected to an electric field. Most of the voltage drop will occur across the plasma membrane. If an electrical potential exists across the plasma membrane, the side facing the anode will be hyperpolarized and that

facing the cathode will be depolarized each by half the total voltage drop across the cell. The presence of the cell will distort the electric field in its vicinity. Therefore, the voltage drop across the cell is not simply the field prorated by the diameter, but will be larger by a factor of about 1.5. The potential at other points on the surface varies as a function of Cos θ. The cytoplasm, by contrast, will be essentially isopotential in spherical, long and thin cells, including neurites or hyphae. However, some voltage drop across the cytoplasm may occur(Peng and Jaffe, 1976; Jaffe and Nuccitelli, 1977; Robinson, 1985). Besides these potential perturbations across the plasma membrane, there occurs a longitudinal gradient of electrical potential along the cell's external surface (Jaffe, 1977; Jaffe and Nuccitelli, 1977). Both of these conditions have biological sequelae.

1.1.8.1 Modulation of membrane potential

Small inequalities in the membrane potential could affect any process sufficiently sensitive to potential changes close to the cell's resting potential. Any change in membrane potential will alter the driving force of ions like Ca^{++} or H^{+} involved in signal transduction across the plasma membrane. But these ions are subjected to large driving forces and their movements are regulated kinetically rather than thermodynamically. More plausible targets of small electric fields are voltage sensitive ion channels, of which the calcium channels are best known (Tsein et al. 1988; Jan and Jan. 1989). Calcium channels in many organisms open in response to rapid depolarization by 15-30 mV. But there do exist calcium channels whose

conductance is regulated by small voltage changes near the resting potential. Thus small inequalities in the local membrane potential could stimulate local calcium fluxes to modulate the physiological processes that underlie growth or behavior.

1.1.8.2 Lateral Electrophoresis of Membrane Proteins

Membrane proteins are known to be mobile in the plane of the lipid bilayer and a significant portion of such protein protrudes into the extracellular matrix (Jacobson and Saier, 1989). These protein are subjected to redistribution by an eiectric field either through electrophoretic or by electro-osmotic mechanism. Jaffe (1977) in his quantitative analysis of this effect, concluded that even small electric fields significantly polarized protein distribution. According to him a voltage drop of 1-5 mV across a cell may achieve one-tenth to one half of the maximal polarization on a time scale of the order of an hour. Possible regulators for redistribution include calcium channels and receptors for growth regulators.

There are reports that a higher field of 1400 mV/mm^2 caused marked redistribution of diverse membrane proteins in the green alga *Micrasterias* accompanied by abnormally thick deposition of cell wall material on the side facing the negative pole (Brower and McIntosh, 1980; Brower and Giddings, 1980). The field strengths required to redistribute membrane proteins are comparable to those found to orient growth and development in *Palvitia.* These

observation has parallel in animal cells where, Con-A show abnormal accumulation under electric field (Poo and Robinson, 1977).

It is surprising that mobile proteins accumulate on the cathodal side, as most membrane proteins are known to be negatively charged and should travel towards the anode. McLaughlin and Poo (1981) resolved the puzzle by taking into account that imposition of an electric field elicits an electroosmotic flow of fluid along the exterior towards the negative pole. Cell surfaces generally bear a negative charge, which is balanced by the accumulation of small cations of the medium. When a field is imposed, these cations will be drawn toward the cathode. Their hydrodynamic drag generates the flow of fluid, which tends to sweep mobile membrane proteins in the same direction. Further these authors argue that the net movement of a mobile macromolecule is a combined function of its zeta potential and that of the surface potential. The zeta potential is the electrical potential at the hydrodynamic plane of shear located about 2 °A from the surface of a phospholipid membrane. A negatively charged macromolecule, whose zeta potential is more negative than that of the surface, will be sweeped by fluid flow towards the negative pole.

1.2 Aims and Objectives

The literature survey reveals an intimate relationship between plants and electricity. The earlier investigations clearly demonstrated that the growing plant

cells and tissues generate electric polarity which enhanced their regulated growth and development. The application of exogenous electric current speeds up the growth rate. However, most of these investigations are on either single celled plants i.e. algae, *in vivo* growing plants or on polarized growth of explants in cultures. Detailed informations on the in vitro unpolarized cell and tissue growth in callus are scanty.

The rapidly increasing industrial biotechnological processes require huge amount of plant tissue biomass and regeneration capacity. The supply of biomass falls far behind the requirement. The production of biomass through present callus culture techniques has two main disadvantages i.e. the growth of the cells and tissue is unpolarized and unregulated with the risks of genetic variations, and the rate of growth is slow. Thus there is a need to develop techniques which can augment and monitor the tissue growth in callus cultures.

Earlier Dutta (1983,1987) reported that the external electromagnetic field alters the natural potential of tissues in *in vitro* conditions. Goldsworthy and Rathore (1985) reported the exogenous electric current augment tissue growth in cultures. These reports do not give the mechanism(s) by which the exogenous electric current reorient, modulate or override the endogenous factors to enhance the tissue growth *in vitro*.

The above issues stimulated the investigator to study the effect of exogenous current on the unpolarized and unregulated growth in *in vitro* callus cultures. The aim of the present investigation is to study the mechanism(s) by which exogenous electric current enhances and regulates the polarized growth of callus *in vitro*. To achieve this objective, following aspects were investigated:

1. Measurement of natural electric potential of non-organogenic, organogenic and embryogenic callus with reference to the culture media, to determine the electric potential distribution and requirement in different tissues during organogenesis of *Nicotiana tabacum* and *Populus deltoides*.
2. Application of 1 μA and 2 μA electric current of each polarity (positive and negative) through the growing tissue with the aim of bringing the negative electric potential to a required potential regime for organogenesis. This is to find out whether or not the exogenous current augments the regeneration and/or growth potential.
3. Cytomorphological analysis of cells of tissue(s) growing under the influence of 1 μA and 2 μA current of each polarity. The cytomorphological analysis includes number of cells per gram fresh weight, ratio and volume of isodiameteric and elongated cells. These analyses should provide clue to the mechanism(s) for cellular polarization.
4. Measurement of the transcellular current before and after passing the electric current in presence and absence of calcium and its inhibitor to determine the dependency of cell polarization on calcium.

Finally attempts are made to find out the overall mechanism(s) by correlating the results of parameters mentioned above.

Chapter 2

Plant Tissue Culture Protocols

The following are the common tissue culture techniques were adopted during the experiments on electrophysiology. The specific experimental design, viz. the measurement of electrical potential in callus and transcellular current in cells, passing electrical current through cultures, and cytomorphological studies are described in respective chapters.

2.1 Experimental Plant Materials

The tissue culture and electrophysiological studies were carried out on following plants:

(a) *Nicotiana tabaccum* commonly known as tobacco

(b) *Populus deltoides* commonly known as poplar

2.1.2 *Nicotiana tabaccum*

The pure line seeds were procured from the Central Tobacco Research Institute, Rajamundary (A.P.). The seedlings were developed and aseptically cultured on White's Medium (composition given in Table 1). These seedlings were used as experimental material in further experiments.

2.1.3 *Populus deltoides*

The source for the explants of popular was the plants grown in the campus of the National Botanical Research Institute, Lucknow. The explants comprised of single node segments. The single node segments (axillary buds) excised from the field plants were surface sterilized and used for establishing the plant material in aseptic culture.

2.2 Surface-sterilization of Explants

Vegetative shoots with axillary buds of about 2.0 cms. in length, were cut into nodal segments each having one axillary bud in case of *Populus deltoides*. For *Nicotiana tabaccum* the seeds were taken as explants. The explants of both the plants were thoroughly washed under running tap water for half an hour then they were pretreated with 5% teepol (liquid detergent) for five minutes by continuously shaking the container. The pretreated nodal segments and seeds were washed in single distilled water and given a quick dip in 95% ethanol followed by immersion in 0.1% mercuric chloride solution for 10 minutes. The nodal segments and seeds were washed with sterile distilled water by giving 4 to 5 washings of 5 minutes each and inoculated on medium separately.

2.3 Culture Medium

2.3.1 Medium Preparation

The Gamborg's (B5), and Linsmair and Skoog's (LS) media were used as basal media (BM) for the culture of *Nicotiana tabaccum* and *Populus deltoides*

respectively. The detailed composition of these media is as given in Table 2.1. Throughout the investigation, all culture propagation, growth and electrical experiments were carried out in the respective medium for each plant.

2.3.2 Medium Preparation Method

The basal medium was prepared in glass doubled distilled water. The basal medium was further supplemented with certain growth regulators depending upon the nature of experiments as given in 2.5. The pH of the medium was adjusted to 5.8 for Gamborg's medium and 6.0 for LS medium using 1N or 0.1 N solution of HCl and/or NaOH before autoclaving. The 0.8% agar was added to the medium and heated till agar melted. The 50 ml. medium is poured in 100 ml Erlenmyer's flask. The culture vessels were plugged with cotton wrapped in gauze cloth and sterilized at 1.08 $kg.cm^{-2}$ in autoclave for 15 minutes.

2.4 Culture Establishment and Maintenance

2.4.1 Establishment of plants in aseptic cultures

The surface sterilized explants were inoculated in the nutrient agar. In case of nodal segments, basal-cut ends were inserted into the agar-nutrient medium under aseptic conditions using a laminar flow. Then cultures were incubated.

2.4.2 Incubation of cultures

The cultures were incubated under 3 kilo lux cool fluorescent light for 14 to 16 hours followed by a dark period of 8 to 10 hours in a 24 hour cycle. The

temperature and relative humidity (RH) of the culture room were maintained at 27 ± 1 °C and approximately 70% respectively. The duration of incubation varied from 21 days to one month according to the experimental requirement.

2.4.3 Maintenance of aseptic cultures

Once the desired growth of the nodal stem-segments achieved and seedlings developed, the shoots were maintained in their respective medium (Table 2.2) by periodic subculture after 4 to 6 weeks intervals at temperature and RH specified in 2.4.2.

2.5 Growth Regulator Supplement in Media

2.5.1 Induction of Undifferentiated growth

The callus were initiated from excised hypocotyls of *Nicotiana tabaccum* and leaves segments of *Populus deltoides* in their respective basal medium containing following growth regulators:

(a) for *Nicotiana tabaccum:* 2 mg/l 2,4-D and 0.3 mg/l kinetin

(b) for *Populus deltoides*: 2.0 mg/l 2,4-D and 0.25 mg/l BAP

2.5.2 Shoot Proliferation and Maintenance

The excised shoots were proliferated, from aseptically grown, seedlings in case of *Nicotiana tabaccum* and axillary buds in case of *Populus deltoides*, were grown on the medium containing 15 mg/l AdS and 1 mg/l IAA, and 15 mg/l AdS, 1 mg/l IAA and 0.25 mg/l BAP respectively.

2.5.3 Differentiation in undifferentiating tissue

The undifferentiated tissues of *Nicotiana tabaccum* and *Populus deltoides* were regenerated on their respective basal medium supplemented with 1 mg/l kinetin and 0.3 mg/l IAA.

2.5.4 Embryo formation (Embryogenesis) in callus

The embryo formation in *Nicotiana tabaccum* and *Populus deltoides* were analyzed in respective BM supplemented with 0.1 mg/l 2,4-D and 0.1 mg/l kinetin followed by respective subculture into medium given under subheading 2.5.2.

2.5.5 Suspension cultures

The suspension cultures of *Nicotiana tabaccum* and *Populus deltoides* were grown in respective basal medium supplemented with 0.1 mg/l kinetin and 1 mg/l 2,4-D. The suspension media was prepared without agar. The cultures after inoculation were incubated over shaker under said environmental conditions.

2.5.6 Medium for Measurement of Transcellular Current:

The measurement of transcellular current in cells is carried out in the same medium as described earlier except that the calcium was not added for calcium devoid experiments. Whereas in some experiments the calcium inhibitor cobalt chloride (2.0 mM) added into the medium with calcium present in the medium to determine the effect of calcium.

Table 2.1: The composition of media
(in mg./l)

Component	B5 Medium[2]	LS Medium[1]	White's Medium[3]
MAJOR SALTS:			
NH_4NO_3	-	1650	-
KNO_3	2500	1900	80
KCl	-	-	65
$CaCl_2.2H_20$	150	440	-
$Ca(NO_3)_2$	-	-	300
$MgSO_4.7H_2O$	250	370	720
KH_2PO_4	-	170	-
$(NH_4)_2SO_4$	134	-	-
$Na_2SO_4.10H_2O$	-	-	200
$NaH_2PO_4.H_2O$	150	-	-
$Na_2H_2PO_4.7H_2O$	-	-	165
MINOR SALTS:			
KI	0.75	0.83	0.75
H_3BO_3	3.0	6.2	1.5
$MnSO_4.4H_2O$	-	22.3	3.0
$MnSO_4.H_2O$	10	-	-
$ZnSO_4.7H_2O$	2.0	8.6	3.0
$CuSO_4.5H_2O$	0.025	0.025	0.01
$CoCl_2.6H_2O$	0.025	0.025	-
Na_2.EDTA	37.3	37.3	-
$FeSO_4.7H_2O$	27.8	27.8	-
$Fe(SO_4)_3$	-	-	2.5

Table 2.1 (Contd.)

Component	B5 Medium	LS Medium	White's Medium
VITAMINS AND ORGANICS:			
myo-Insitol	100	100	-
Nicotinic acid	1.0	-	0.05
Pyridoxin HCl	1.0	-	0.01
Thiamine HCl	10	6.4	0.01
Glycine	-	-	3.0
Ca-D-panthothenate	-	-	1.0
Sucrose	20g	30g	20g
pH	5.6	6.0	5.8

[1] Linsmair and Skoog medium (1965)
[2] Gamborg B-5 medium (1968)
[3] White's modified medium (1994)

Chapter 3

Electrical Control of Differentiation in Callus Natural Potential

3.1 Introduction

The induction and maintenance of physiological and morphological polarities involve the passage of natural electric currents through the cells concerned (Jaffe and Nuccitelli, 1977; Quatrano, 1978; Schnepf, 1986). When organized polar structures are formed, their component cells normally share the same electrical polarity and grow in the same direction. This was demonstrated by Lund (1947) for individual cell of filamentous alga *Pithophora.* This has also been found true for the longitudinal potential gradients developed along higher plant organs such as cereal coleoptiles (Lund, 1947) and developing carrot embryos (Brawley et al., 1984).

Rathore & Goldsworthy (1985) has suggested that the disorganized growth of differentiated tissue cultures might be due to the lack of a common polarity in their component cells and with the vibrating probe on tobacco cells grown in suspension culture shows only a partial coordination of polarities (Goldsworthy and Mina, 1991). Growing cells in cultures exhibit visible response with different growth factors, cells growing in an IAA-containing culture medium conducive to differentiation, tended to form more or less straight filaments in which the electrical axis of the component cells were normally parallel with the

axis of growth, but their polarities may not always be in the same direction. Cells growing in a medium containing 2,4-D, which was not conducive to differentiation, were much more disorganized as they formed random clusters rather than straight filaments. Further the component cells had unstable electrical polarities due to the slowly drifting points of current entry and exit over the cell surface. It was argued that this instability prevented the formation of straight filaments and might also prevent differentiation into any organized structure based on linear columns of cells (Nuccitelli, 1978; Quatrano, 1978).

It is, therefore, expected that the differentiation of tissue cultures to be associated with a greater coordination of cell polarities and corresponding increases in bioelectrical potentials for the tissue as a whole, as more and more cells align their electrical axes in a common direction.

The present work set out to study differentiating *Nicotiana tabacum* and *Populus deltoides* callus to see if there were any corresponding electrical changes during "embryogenesis" and "organogenesis" to form shoots. The measurement of the surface potentials of callus were made to see if there was any electrical patterns associated with their differentiating regions. The comparison in the behavior of developing embryoids between the calli has also been attempted.

3.2 Materials and Methods

3.2.1 Tissue Culture

Tobacco callus was established from hypocotyls of *Nicotiana tabacum* and leaves of *Populas deltoides* using tissue culture techniques as described in

chapter 2. The cultures were maintained at 25 °C under 3.0 mol.m.$^{-2}$sec.$^{-1}$ fluorescent illumination for 5-6 weeks to initiate callus growth. Unless otherwise stated, the callus was subcultured by transferring actively growing portions weighing approximately 500 mg. every 5-6 weeks onto a similar medium. Shoot-formation in *Nicotiana tabacum* and *Populus deltoides* was obtained by subculture onto Gamborg's and LS medium respectively in which the kinetin was increased to 1.0 mg.l^{-1} and the 2,4-D replaced by 0.03 mg.l^{-1} indole acetic acid (IAA). Embryo-formation in *Nicotiana tabacum* and *Populus deltoides* was obtained by subculture onto a medium containing 0.1 mg.l^{-1} 2,4-D and 0.1 mg.l^{-1} kinetin followed by subculture onto a normal auxin (IAA) containing medium (Table 2.1). In all cases, 100 ml of flask was used as the culture vessels to facilitate the application of the electrodes.

3.2.3 Electrical measurements

Silver/ silver chloride electrodes were made from 40 mm lengths of 0.125 mm diameter silver wire. Several electrodes were made simultaneously by putting the silver wire (s) as anodes in an electrolytic cell containing 1.0 mM KCl. A longer length of silver wire served as the cathode (Fig.3 A). A potential of 1.0 volt was applied to the cell for 18 hours in the dark, causing a layer of white silver chloride to be deposited on the anodes. The chloridized anodes were then removed and mounted for use as the measuring electrodes. Each electrode was sealed into a flexible plastic pipette tip, filled with 3.0 M KCl, using hot adhesive (Solvite S-1) from a glue gun (Fig. 3 B). After removing the extreme end of the

tip with a razor blade to give a tip diameter of approximately 1.0-mm, each pipette tip was fastened to the end of a short autoclaved wooden rod mounted in a micromanipulator. The silver wire of the chloridized electrode was then soldered to a screened lead for connection to the measuring equipment. Before use, the electrodes were stored as pairs in the dark (silver chloride is decomposed by light) with their tips in 3.0 M KCl and their terminals connected together to minimize the development of stray electrode potentials.

Measurements of surface potentials were made with one electrode from the pair embedded in the culture medium and the other gently touching the surface of the callus. Where repeated measurements were required, the whole electrode assembly, including the micromanipulator, was mounted on a continuously running laminar-flow bench and sterilized by swabbing with 70 percent ethanol. This method was found very good to takeseveral measurements on the same culture over a period of several weeks without contamination.

In some experiments, voltages were measured using a purpose-built high impedance DC preamplifier (input impedance circa 10^{12} ohms) connected to a chart recorder. In other experiments, measurements were made directly with Backman HD153 digital multimeter (from RS Components, Corby, UK). Despite its lower input impedance (10^{7} ohm) it gave an audible signal, the pitch of which corresponded to the voltage. This allowed the operator to concentrate on probing the tissue with the electrode, to monitor the audible changes in pitch when contact had been made with the callus to identify the position of peak values without constantly referring to the visual display. The monitoring of electrical potential

was carried on undifferentiated as well as differentiating callus. For this purpose both types of callus were divided into central, intermediate and outer regions/ zones.

3.3 Results

Both undifferentiated and differentiated calli appeared to be thickest at the center and thinner at the outer region. The observations on surface potentials of each region are given in Table 3.1.

3.3.1 Surface potentials of undifferentiated callus

When the surface potentials of undifferentiated callus cultures were measured at various zones in 25 replicate cultures, it was found that they were always negative to the culture medium, with the values tending to be highest at the central regions of the calli of both *Nicotiana tabacum* and *Populas deltoides* as is evident from Fig.3.1 and 3.2 and Table 3.1.

The results obtained were found to be the same regardless of the species of callus and the culture medium used, or the auxin supplied. The results shows typical values (Table 3.1 and Fig. 3.1 and 3.2) obtained from mature tobacco and popular callus growing on undifferentiating media (Chapter 2). A precise statistical analysis of the results is difficult because of variations in size and shape of the cultures and the problem of making measurements at defined locations on amorphous replicates. However, the surface potentials were always relatively low. Form several hundred measurements made on a total of 25 cultures, they were

typically less than 10.0 mV, never exceeded 15.0 mV and there were no sharp discontinuities.

3.3.2 Surface potentials of differentiating callus

3.3.2.1 Organogenesis

When tobacco callus was transferred to the shoot-forming medium under illumination, small green areas with densely packed cells appeared (Plate 3.1). They arose from regions rich in meristemoids and it is these structures which eventually gave rise to the shoots. The appearance of the green areas coincided with marked electrophysiological changes. The green spots developed remarkably high surface potentials, typically 20-80 mV negative to the culture medium, whereas the intervening undifferentiated regions of the callus remained low, usually less than 10.0 mV (Table3.1 and Fig.3.1 and 3.2). The electrical boundary between the green and the non-green areas was extremely sharp, the change occurring in less than a millimeter distance (which was the limit of resolution of the electrode) and may have been of cellular dimensions. This suggests that the meristemoids are electrically as well as physiologically distinct from the undifferentiated pseudoparenchyma of the surrounding callus. However neither the shoots or the callus were now green, although the developing meristemoids were visible as areas of small cells with surface potentials nearly as high as their green counterparts.

The microscopic studies show these regions to consist of parallel rows of cells, suggesting that they have a stronger and more coordinated anatomical polarity than the more randomly growing cells around them.

3.3.2.2 Embryogenesis

When *Populus deltoides* callus was transferred to media which induce embryoid formation, there were no electrical changes corresponding to those seen during its organogenesis. Embryogenesis occured in *Populus deltoides* calli but not in *Nicotiana tabacum*. Thus embryoid formation in callus does not appear to depend on the presence of an electrically distinct stem like structure. The present experiment shows that a steep increase or decrease of potential gradient in the surrounding callus is not prerequisite for the embryo formation.

3.4 Discussion

Earlier reports with carrot embryos developing in culture indicate an increase in electrical polarity corresponding to the time when the structures show visible polar growth (Brawley et al., 1984). Mina and Goldsworthy (1991) showed that one of the first visible signs of differentiation in tobacco callus was the polar growth of vascular nodules (meristemoids) embedded near the surface to form elongated tracts of cells with a parallel orientation. This was followed by the emergence of shoots from the sides of these structures. These potentials, appeared to be of metabolic origin since they had a Q_{10} of approximately 2 over the range 25-35 °C, were reduced in brown moribund regions, disappeared when the callus was heated to 50 °C and were absent in old dead cultures. The callus is composed of successive layers of cells between the culture medium and the surface. The electrical pattern obtained may be attributed to the callus cells having

a weak but predominantly vertical electrical polarity with their upper extremities tending to be negative. Each layer being electrically in series with those above and below, contribute to the overall voltage, with the thicker parts giving higher values because there are more layers to contribute.

This suggests that the meristemoids are electrically as well as physiologically distinct from the undifferentiated pseudoparenchyma of the surrounding callus. The electrical difference between the green meristemoid-rich regions and the surrounding tissue has been reported to be not due to the ability of the meristemoids to photosynthesise (Machackova and Krekule, 1991). When callus was grown in the dark, shoot-formation still occurred. However neither the shoots nor the callus were now green, although the developing meristemoids were visible as areas of small cells with surface potentials nearly as high as their green counterparts.

The microscopic studies show these regions to consist of parallel rows of cells, suggesting that they have a stronger and more coordinated anatomical polarity than the more randomly growing cells around them.

If this corresponds to a greater degree of electrical polarity, then the cumulative effect would generate the higher potentials observed on the callus surface above. The close juxtaposition of the high potential of the meristemoid surface and the low potential of the surrounding callus surface indicate the presence of a steep lateral voltage gradient between the two. Since artificially applied potential gradients have been reported to repolarize tobacco cells, perhaps

this lateral gradient repolarizes the cells at the periphery of the meristemoid to form the buds that then grew out laterally (Mina & Goldsworthy, 1991)

A working hypothesis for the electrical control of organogenesis might be that in the 2,4-D medium, partially structured meristemoids may arise, but are unable to elongate because the medium does not permit the maintenance of stable cellular polarity. These structures contain vascular tissue and bear some anatomical resemblance to growing stems embedded in the callus. They also have similar electrical characteristics i.e. a strong electrical polarity with the upper part negative. Perhaps buds arise on the surface of these 'stems', using the same electrical clues as they would on the real stems of an intact plant.

The surface potential of the callus remained uniformly low, even in areas where large numbers of embryoids themselves become strongly polar as they develop (Brawley et al., 1984). The electrical conditions in the callus needed for organogenesis.

Embryoid formation in callus does not appear to depend on the presence of an electrically distinct stem like structure. When popular callus was transferred to media which induced embryoid formation, there were no electrical changes corresponding to those seen during tobacco organogenesis. The surface potential of the callus remained uniformly low, even in areas where large numbers of embryoids were being formed. Although the embryoids themselves become strongly polar as they develop (Brawley et al., 1984), the present experiment shows that steep potential gradient in the surrounding callus is not a prerequisite for their formation. The electrical conditions in the callus needed for

organogenesis seem to be quite different to those for embryogenesis. Buds, which normally grow as an integrated part of an electrically polar stem, like structures to establish their own polarity and orientation. Conversely, embryos, which are essentially separate plants, appear to need no electrical cues from the surrounding tissue and develop their own polarity unaided.

Table- 3.1 Surface Potential of Callus in *Nicotiana tabacum* and *Populus deltoides* (average of 25 replicates)

Region On Callus	Surface Potential (in mV)			
	Nicotiana tabacum		*Populus deltoides*	
	Undifferen. Medium	Differen. Medium	Undifferen. Medium	Differen. Medium
Outer	8.0 ±0.73	71.5 ±2.47	10 ±1.06	100.4 ±4.65
Intermediate	4.4 ±0.40	44.8 ±3.91	7.2 ±0.36	51.6 ±4.13
Central	2.1 ±0.44	22.6 ±1.71	2.30 ±0.28	30.8 ±3.56

Fig.-3.1 Surface Potential of Callus in *Populus deltoides*

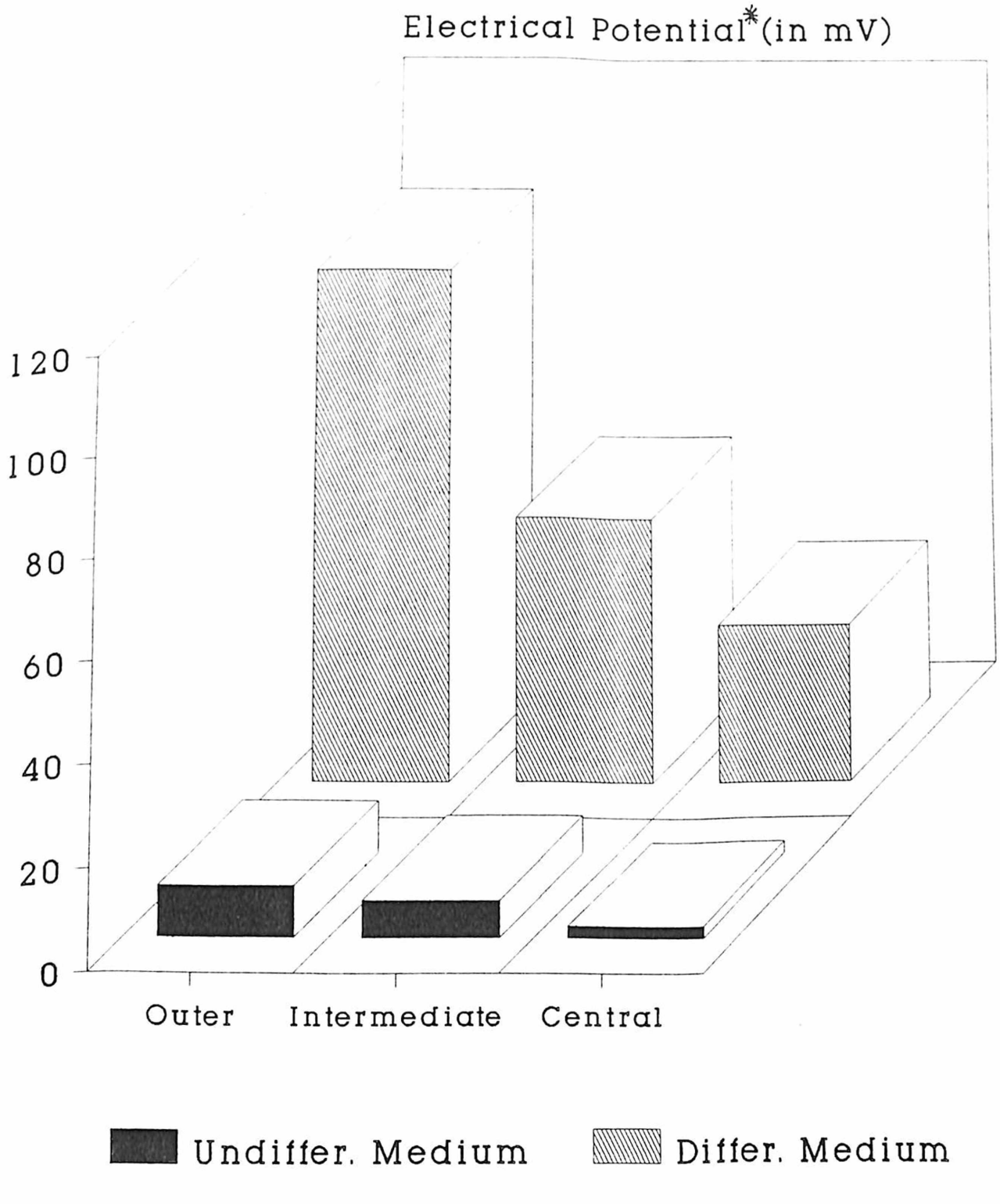


Fig.-3.2 Surface Potential of Callus in *Nicotiana tabacum*

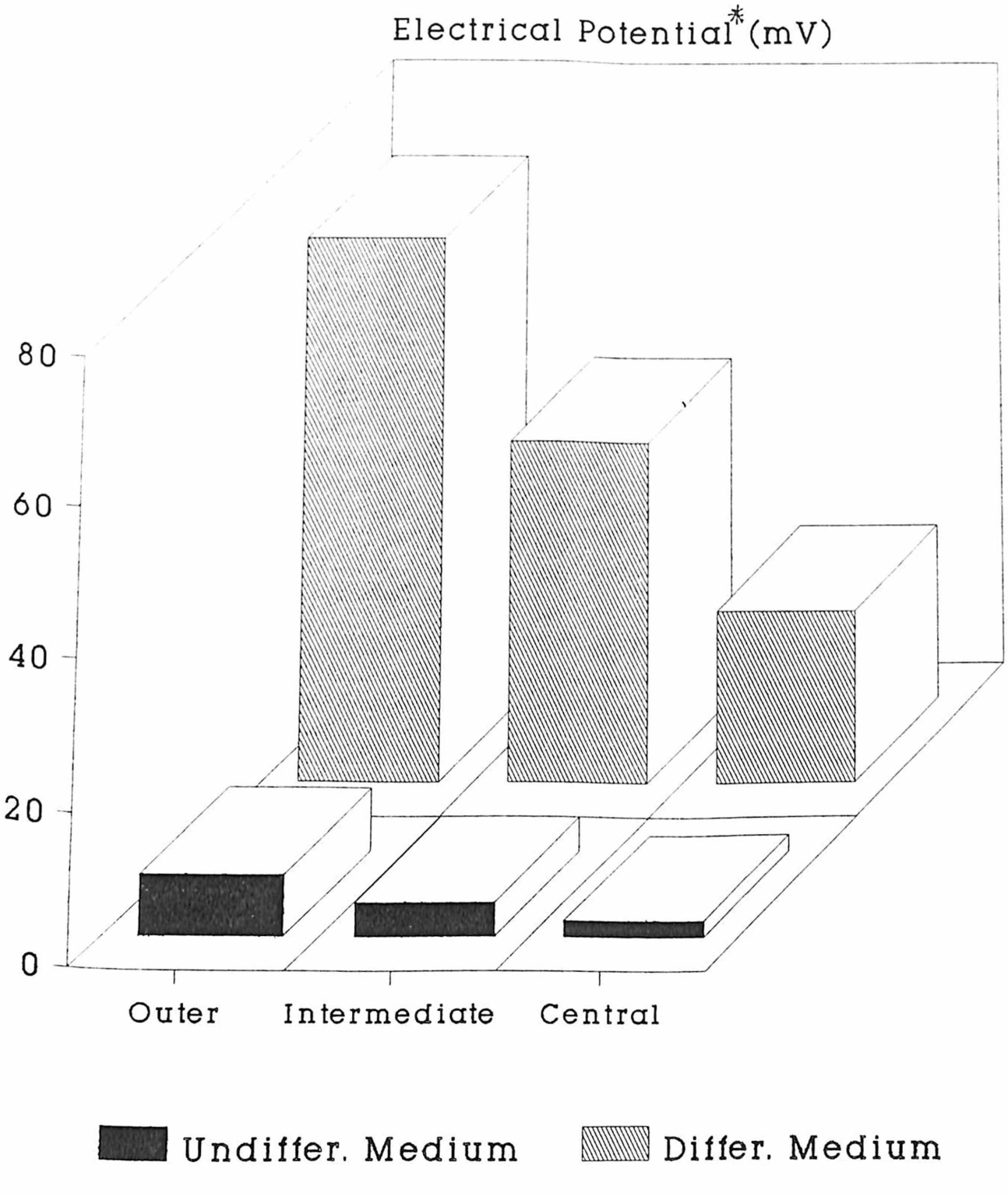


Fig.3A Preparation of Ag/AgCl electrodes

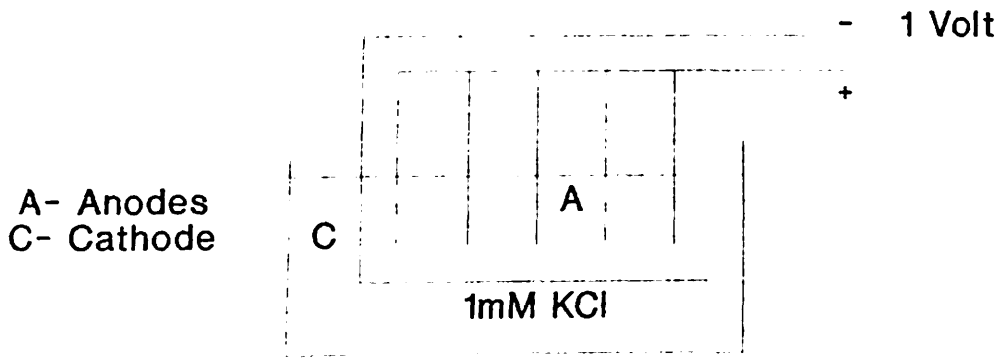


Fig. 3B A Diagram of Electrode assembly

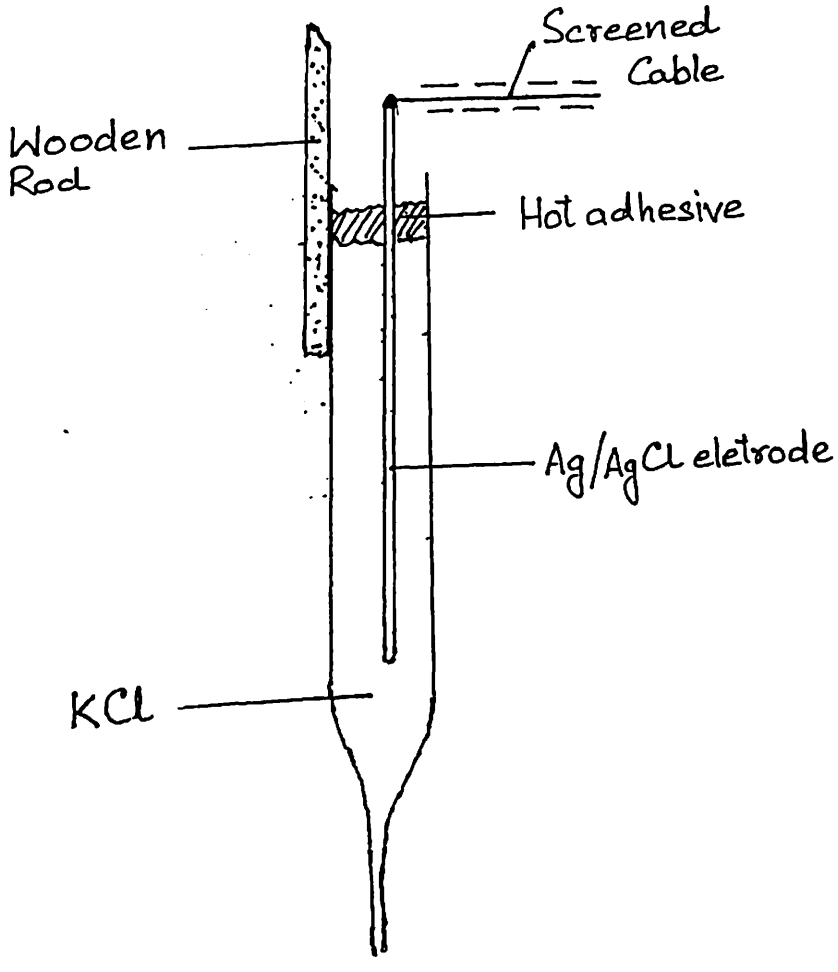
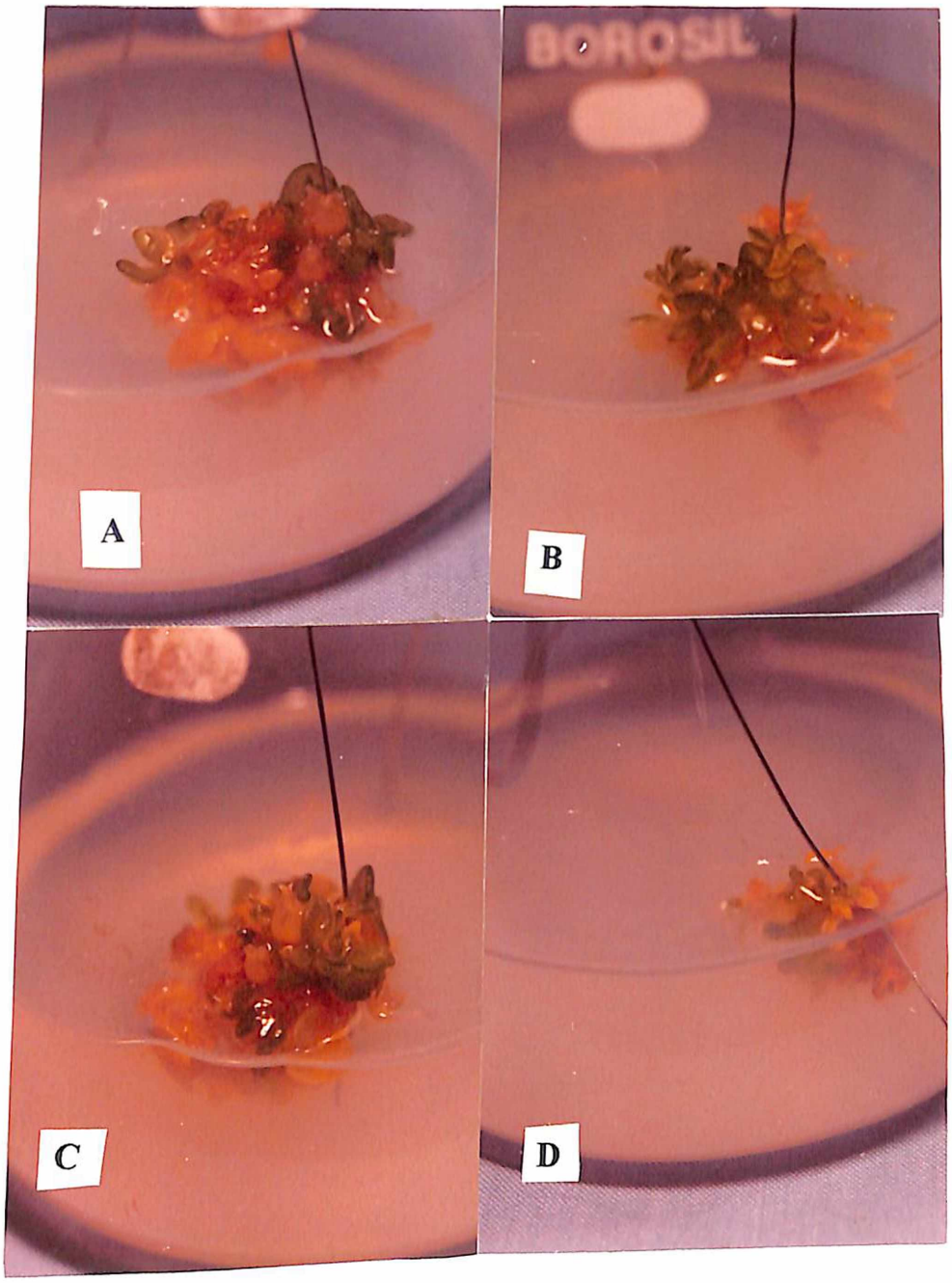


Plate 3.1: Regeneration in "callus negative" treatments
Green regenerating zones are clearly visible

A- in *Nicotiana tabacum*
B- in *Populus deltoides*
C- in *Populus deltoides*
D- Regeneration in "callus positive" in *Nicotiana tabacum*
Green regenerating zones are visible in callus near the medium


A
BOROSIL
B
C
D

Chapter 4

Electrical control of growth in plant tissue culture

4.1 Introduction

There is a large and growing number of reports in the literature on the control of cellular polarity by weak electric currents which flows through the cells, viz. unicellular algae (Jaffe & Nuccitelli, 1977), the eggs of seaweeds (Lund, 1947) and eggs of both vertebrates and invertebrates (Jaffe & Nuccitelli, 1977) etc. These currents, which have densities of the order of microamperes per square centimeter, are generated by asymmetries resulting from uptake and expulsion of various ions along the cell axis. Such electrical polarity has also been reported in multicellular algal filaments (Bethmann et al., 1995) and cereal coleoptile (Thiel et al.,1994). The electrical polarity in plant tissues is correlated with their physiological polarity and give rise to longitudinal voltage gradients of the order of tens of millivolts per centimeter along their axes of growth (Lund, 1947; Newman, 1963).

The polarity of single cells can be controlled by externally applied electrical fields as in the case of the *Fucus* egg (Lund, 1923) and *Acetabularia* stalks (Novak & Bentrup, 1972). In multicellular structures, interference with natural voltage gradients by artificially applied currents stimulates the rate of shoot growth when applied longitudinally (Black et al., 1971), but the direction of growth changes when applied transversely (Webster & Schrank, 1953). A possible explanation for these results is that the applied current either enhances or

redirects the natural polarity of the cells concerned. Though effect of externally applied current is now known on the growing tissues/cells having natural polarities, but little is known about the effects of external current in tissues having no natural polarity. Therefore in the present investigation the effects of similar currents on callus cultures which have no obvious natural polarity were studied. The results were surprising and possibly of considerable value to the biotechnologists.

4.2 Material and Methods

Callus cultures were initiated in aseptically grown seedlings of *Nicotiana tabaccum* and *Populus deltoides* (as described in Chapter 2). Each experiment was conducted on a single clone callus produced in three to four week time under weak illumination. The callusing media was supplemented with respective growth regulators (as mentioned in 2.5.1) to provide a homogenous callus with suppressed differentiation. The inocula for each experiment, weighing between 140 - 175 mg., were cut from actively growing regions of callus, and placed on the experimental media in 100 ml. conical flasks. The electrodes of 0.25 mm diameter and 15-cm long stainless steel wire, were sterilized by autoclaving before use. One electrode was inserted about 2.0 mm deep into the center of the callus and the other into the medium about 20.0 mm away. The wires were bent over the rim of the flask, which was then covered with cotton plug and aluminum foil. Current of 1 μA and 2 μA were applied continuously from a common D.C. power supply via a separate 10 megaohm resistor to each replicate callus. The

very high value of the resistor than that of the callus ensured a steady current supply throughout the experiment despite changes in tissue resistance. The current was set by adjusting the voltage of the power supply and checked at intervals throughout the experiment by an Ohm's law calculation from the voltage drop across the resistor measured with digital voltmeter. In one set, the positive electrode was implanted in the callus and other set the negative electrode was implanted in the callus. Accordingly the callus are referred to as 'callus positive' and 'callus negative' treatments respectively. Twenty five replicate flasks were set up for each treatment together with a same number of controls in which the electrodes were implanted but not connected to the power supply. The cultures were maintained at 27±1 °C with 16 hours photoperiod under uniform diffuse fluorescent illumination of 3.0 lux.

4.3 Results

4.3.1 Effects on Undifferentiated growth

Undifferentiated growth was obtained by growing the experimental cultures on a culture medium containing 0.3-mg/l kinetin and variable concentration of IAA or 2,4-D. Two current levels of 1 μA and 2 μA were tested at each polarity in the experimental replicates. The control replicates were simultaneously maintained without current supply.

The culture in media contained 4.0 mg/l IAA and with 'callus negative' treatments, were growing noticeably faster than the controls by the 10th day both in *Nicotiana tabaccum* and *Populus deltoides*. After 4 weeks, the cultures were

harvested and their fresh and dry weights were determined. The results of these experiments are as recorded for *Nicotiana tabaccum* (Table 4.1 & Fig 4.1) and *Populus deltoides* (Table 4.1& Fig 4.7). The similar results were observed with higher concentration IAA (i.e 27 mg/l) in both *Nicotiana tabacum* (Table 4.2 and Fig. 4.2) and *Populus deltoides* (Table 4.2 and Fig. 4.8). On the contrary, the results of 'callus positive' treatment exhibit either normal or slightly inhibited growth as compared to the controls for both *Nicotiana tabaccum* (Table 4.1 & Fig 4.1) and *Populus deltoides* (Table 4.1& Fig 4.7). A comparison of growth rate reveals that 'callus negative' cultures grow about 60% faster than both 'callus positive' and control cultures (Plate 4.1).

These results indicate that the electric current had possibly polarized the cells of the callus along with its direction of flow, resulting in the polar transport of auxins through the culture. With the current in one direction, auxin appears to be actively transported into the tissue giving increased growth. But with the reverse in current direction, the cellular polarities were reversed with no polar auxin uptake, and growth remaining similar to the controls.

To test the above conclusion, a similar experiment was performed with medium without IAA. The results can be seen in Tables 4.3 Figs. 4.3 and 4.9. Although the growth pattern of the cultures was almost similar to those with IAA, presumably due to endogenous auxin carried over the previous subculture, there was no significant electrically stimulated growth. Thus, the results suggest that the effect was due to the polar uptake of auxin under the influence of exogenous electric current.

As a further test of this hypothesis, the experiment was repeated with IAA at concentration as before but in the presence of 27.0 mg/l TIBA, which is an inhibitor of the polar transport of auxin (Niedergang-Kamien and Skoog, 1956). The results are shown in Table 4.4 and, Fig. 4.4 and 4.10. The culture grew reasonably well, but not as fast as was the case in the absence of TIBA. The electrical stimulation was again found to be absent confirming that it was not due to a general inhibition of growth in the presence of TIBA. The experiment was performed in absence of TIBA and IAA replaced by 1.0 mg/l 2,4-D. The auxin 2,4-D is not thought to show significant polar transport. The results are shown in Table 4.5 and, Fig. 4.5 and 4.11. It is evident from the results that the cultures grew well in 2,4-D but there was still no electrical stimulation of growth. This confirms the conclusion drawn from the present observation that the electrical effect on callus growth is due to polar uptake of auxin i.e. IAA.

4.3.2 Effect on shoot regeneration

In the previous experiments, the culture media were formulated to suppress differentiation, but there was a tendency for the electrically treated cultures to be greener and to show the beginnings of shoot regeneration. To investigate this, the experiment was repeated using a culture medium containing 15.0 mg/l AdS, 1.0 mg/l IAA and 0.25 mg/l BAP, which is more favorable to shoot formation. The cultures were examined at regular intervals and the number of shoots, which had formed, was recorded.

Table 4.6 and, Fig. 4.6 and 4.12 show the results of two separate experiments viz. negative and positive callus. Although they were performed with different clones, with the quite different 'natural' regeneration potential, electrical treatment brought about 5 to 8-fold stimulation of regeneration in both *Nicotiana tabaccum* and *Populus deltoides* (Plate 4.2). In contrast to the undifferentiated growth, the regeneration was electrically stimulated by currents of both polarity, although there was a tendency for the effect to be greater in the 'callus negative' treatments. In experiments, where the cultures had the higher morphogenetic potential, all electrical treatments were significantly different from the controls (at both the 0.01 and 0.001 levels). In experiments, where the numbers of shoots were generally much lower, the percentage stimulation was approximately the same for both the polarity. The pooled data for both current levels showed them to be statistically significant at $p< 0.01$, $p<0.001$ and $p< 0.05$ for the 'callus-negative' treatments.

Although shoots eventually formed all over the callus, there was a tendency for the first one to arise in the most negative region of the callus, i.e. near the electrode at the tip of the callus when this electrode was made negative, or at the base of the callus near the medium when the medium was made negative. A possible explanation is that the 'polar' transport of IAA towards the negative electrode made growth occur faster in this region.

4.4 DISCUSSION

The present investigation demonstrates that normal callus growth occurred even in media lacking an auxin. The growth rate remained unaffected by application of electrical current. Further, the growth became enhanced by electrical application if IAA was added to the culture medium. This suggests that the applied current may stimulate growth by effecting the IAA uptake. It cannot be due to the simple electrophoretic uptake of (IAA) ion, because the growth stimulation occurred only when the callus was negative to the medium [the negative (IAA) ion should therefore have been repelled]. It could nevertheless be explained by assuming that the electric current entrained the physiological polarities of the callus cells along with its direction of flow to enable the polar transport of auxin. Thus when the current is passed from medium to callus, auxin would be drawn into the callus by the polar transport mechanism to stimulate growth, but auxin tend to move out of the callus with the reversal of current therefore the growth would be little different from that in the controls.

This conclusion is further supported by the fact that TIBA (an inhibitor of polar transport) and the substitution of IAA by 2,4-D (an auxin showing little polar transport) both abolished the electrical effect.

The above observations are in conformity with the earlier observations that natural currents normally flow through animal and plant cells to control their polarity (Jaffe & Nuccitelli, 1977). Lund (1947) examined the mechanism by which the current is generated in the Fucoid egg. He found that the current is driven by ion pumps and passive leakage channels at opposite ends of the cell

which intern cause a unidirectional circulatory current flow from the cytoplasm to the medium. Similar observations has also been made on the currents flow in multicellular structures such as carrot embryoids (Brawley et al., 1984), cereal coleoptiles and the filaments of the alga *Pithophora* (Lund, 1947). In these cases, the currents follow a multicellular pathway, which may provide the common frame of reference necessary for the coordinated polar growth of organized structures.

The mechanisms by which natural current control polarity is still not fully understood and many possibilities have been suggested. One possibility is by the localized entry of calcium ions. It has been shown in the fucoid eggs that a small but significant part of the current is carried by calcium ions entering the sites of polar growth. The calcium ions activate a whole array of enzymes, some of which could be responsible for localized growth in this region (Robinson & Jaffe, 1975). Another possibility suggested is that as the current flowing through the cell, bring about the electrophoretic movement of charged substrates and charged vesicles containing substrates towards the polar growing regions (Jaffe et al, 1974). Although both of these hypotheses provides an explanation for the polar growth of tip-growing cells, they do not account for events affecting differentiation elsewhere in the cell such as the equatorial location of the cell division.

A possible explanation for the above hypothesis may be based on the observation of Poo and Robinson (1977) on the behavior of directly charged membrane proteins. The proteins can move by lateral electrophoresis in the fluid mosaic of the cell membrane. Such proteins are constrained to remain within the

membrane by their lipid solubility but will be attracted towards one or other of the electrical poles of the cell, depending on their charge. Highly charged proteins will jostle most of their way along the membrane to the poles, and force less highly charged ones to take up equilibrium positions closer to the cell's equator. The membrane may therefore contain bands of differently charged proteins at positions along the cell's electrical axis corresponding to their charge. Since these proteins may have enzymatic activity or structural importance, specific structures or patterns of metabolism could be located at precise positions along the cell axis by this means. It is also possible that a similar mechanism may determine the location of the non-membrane proteins in those regions of the cell which are not affected by cytoplasmic streaming such as in the stationary ectoplasm.

Such electrophoretic location of specific proteins may also provide a mechanism for cell. For example, when a meristematic cell divides, each daughter would contain a different complement of proteins (again based on their charge) which in turn could activate different components of the genome so that one cell remains meristematic and the other differentiates to form mature tissue.

Lateral protein electrophoresis may also account for the occurrence of mixtures of many electrophoretically separable isoenzymes, required for uniform activity along the whole of the cell's axis. It also give added significance to observations that different tissues frequently have different isoenzymes patterns, since particular forms of differentiation may require specific non-uniform enzyme locations (Poo and Robinson, 1977).

This hypothesis, possibly for the first time, provide explanation of how the genetic code of DNA determines not only the structure and activity of proteins, but affect their intracellular distribution by controlling their charge. This in turn could control the cell shape and pattern of division, and ultimately the shape and structure of the whole organism. Perhaps the apparently non-functional amino acids in proteins and enzymes, which are not conserved from species to species, have more significance than hitherto thought. By contributing the precise charge to the protein, they may determine the organism's structure and possibly even differentiation of species (Poo and Robinson, 1977).

Whatever the mechanisms by which electric currents affects cells it is increasingly clear that the flow of natural electric currents through living cells plays an important part in determining their polarity and consequently the growth pattern of the whole organism. The present work with tissue cultures seems to indicate that it is possible to control cellular polarity by artificially applied electric currents and their growth as reported in *Fucus* egg (Lund, 1947).

An important question is by what mechanism does an ***externally applied*** current affect cellular polarity in tissue cultures? It is certainly not by its passing through the cell as a substitute for the natural current because the resistance of the membrane is so high that virtually all of the current applied will flow outside the cell walls as reported earlier in *Chara* (Chilcott and Coster, 1991). Instead it appear to entrain the cell's own electric current by relocating the current generating ion pumps and channels proteins. These proteins pass right through the

membrane and have charge on their external surfaces to enable them to move in the applied field and drag the cell's own electrical axis into line with it.

According to Goldsworthy (1986) this sensitivity to external electric fields is a natural mechanism for coordinating cellular polarities in organized structures. If, for example, the ion pumps or channels responsible for making one end of the cell negative were themselves negatively charged, they would be attracted to the positive region of neighboring cells. Similarly, if the pumps or channels making the other end of the cell positive were themselves positively charged, they would be attracted to the negative region of neighboring cells. The effect would be that the electrical polarities would line up the cell nose to tail in parallel rows like iron filings in a magnetic field, with the division of the zygote or whenever a new growth axis is produced. Each cell would then share and coordinated by the common electric current and the aberrant cells will be pulled into line by their orderly neighbors.

In tissue cultures the explants are not under the electrical control of the mother plant and its cells looses their natural coordinated polarities. These explants grow rapidly under growth hormone in non-polarized manner. The present experiments suggest that the polarity can be restored, at least in part, by the artificial application of an electric current of a magnitude similar to the natural one. In these experiments, the apparent direction of auxin flow was as expected from the hypothesis discussed above. In the intact plant, the base is normally electropositive to the apex (Lund, 1947; Newman, 1963), i.e. auxin flow is towards the positive end of each of its component cells. This positive end would

be normally attracted to the negative end of the cell beneath. When cells orientated in an ***artificial*** field, their positively charged bases should rotate towards the negative electrode so that the 'polar' flow of auxin would now be towards this electrode. This would account for the enhanced growth of the cultures when they were made negative to the medium and also the apparent lateral movement of auxin towards the negative electrode when transverse currents were applied to the cereal coleoptile (Webster & Schrank, 1953).

If the rotation of the electrically induced polarity in callus cultures is accepted, it is relatively easy to account for the enhanced level of shoot regeneration. Under normal circumstances, cell division in callus cultures is disordered due to a lack of well-defined current pathways to coordinate cellular polarity. However, from time to time, neighboring cells, by chance, acquire a common polarity, and give rise to the ordered meristems needed to produce organs such as roots and shoots. An artificially applied electric current orienting the polarities of the adjacent cells more or less parallel with one another would increase the statistical probability of clusters of coordinated cells to produce organ-forming meristems. Currents applied in either direction would be effective since both would give the required parallel orientation. A better performance of the callus-negative treatments in the present experiments may be because a rapid uptake of auxin in these treatments had an additional stimulating effect on growth.

Table 4.1 Effect of Passing Electric Current on *Nicotiana tabacum* and *Populus deltoides* callus in presence of 4 mg/l IAA after 30 days of inoculation (average of 25 replicates)

	Increase in Fresh/ Dry weight (in gms.)			
	Nicotiana tabacum		*Populus deltoides*	
Treatments	Fresh wt.	Dry wt	Fresh wt.	Dry wt.
Control	2.58 ±0.48	0.315 ±0.04	2.95 ±0.62	0.18 ±0.03
+ve 2 μA	1.87 ±0.28	0.203 ±0.08	2.63 ±0.38	0.13 ±0.01
+ve 1 μA	2.12 ±0.17	0.288 ±0.09	3.4 ±0.83	0.26 ±0.04
-ve 2 μA	4.67[1] ±0.73	0.581 ±0.27	6.32[2] ±0.58	0.27 ±0.01
-ve 1 μA	4.92[1] ±0.23	0.483 ±0.15	7.13[2] ±0.61	0.34 ±0.05

[1] Significant at $p<0.01$
[2] Significant at $p<0.001$

Table 4.2 Effect of Passing Electric Current on *Nicotiana tabacum* and *Populus deltoides* callus in presence of 27 mg/l IAA after 30 days of inoculation (average of 25 replicates)

	Increase in Fresh/ Dry weight (in gms.)			
	Nicotiana tabacum		*Populus deltoides*	
Treatments	Fresh wt.	Dry wt	Fresh wt.	Dry wt.
Control	2.16 ±0.58	0.29 ±0.11	3.22 ±0.65	0.30 ±0.10
+ve 2 μA	2.08 ±0.18	0.23 ±0.06	3.24 ±0.13	0.35 ±0.08
+ve 1 μA	2.14 ±0.36	0.38 ±0.02	3.19 ±0.20	0.28 ±0.03
-ve 2 μA	4.1[1] ±0.54	0.53 ±0.17	7.62[1] ±1.08	0.63 ±0.20
-ve 1 μA	3.78[1] ±1.22	0.48 ±0.05	8.15[1] ±0.53	0.94 ±0.17

[1] Significant at $p<0.05$

Table 4.3 Effect of Passing Electric Current on *Nicotiana tabacum* and *Populus deltoides* callus in absence of IAA after 30 days of inoculation (average of 25 replicates)

	Increase in Fresh/ Dry weight (in gms.)			
	Nicotiana tabacum		*Populus deltoides*	
Treatments	Fresh wt.	Dry wt	Fresh wt.	Dry wt.
Control	2.04 ±0.37	0.29 ±0.05	2.465 ±0.33	0.239 ±0.17
+ve 2 μA	1.86 ±0.28	0.13 ±0.10	2.18 ±0.15	0.213 ±0.08
+ve 1 μA	2.04 ±0.74	0.27 ±0.03	2.48 ±0.51	0.250 ±0.07
-ve 2 μA	2.05 ±0.26	0.31` ±0.04	2.47 ±0.12	0.235 ±0.03
-ve 1 μA	2.18 ±0.33	0.38 ±0.14	2.49 ±0.39	0.232 ±0.16

Table 4.4 Effect of Passing Electric Current on *Nicotiana tabacum* and *Populus deltoides* callus in presence of 4 mg/l IAA and 27 mg/l TIBA after 30 days of inoculation
(average of 25 replicates)

	Increase in Fresh/ Dry weight (in gms.)			
	Nicotiana tabacum		*Populus deltoides*	
Treatments	Fresh wt.	Dry wt	Fresh wt.	Dry wt.
Control	1.4 ±0.25	0.15 ±0.02	1.295 ±0.21	0.106 ±0.17
+ve 2 μA	0.92 ±0.16	0.18 ±0.05	1.04 ±0.46	0.184 ±0.14
+ve 1 μA	1.32 ±0.28	0.15 ±0.01	1.38 ±0.17	0.113 ±0.10
-ve 2 μA	1.3 ±0.61	0.14 ±0.03	1.405 ±0.19	0.21 ±0.07
-ve 1 μA	1.4 ±0.38	0.20 ±0.07	1.43 ±0.25	0.238 ±0.10

Table 4.5 Effect of Passing Electric Current on *Nicotiana tabacum* and *Populus deltoides* callus in presence of 1 mg/l 2,4 –D after 30 days of inoculation (average of 25 replicates)

Treatments	Increase in Fresh/ Dry weight (in gms.)			
	Nicotiana tabacum		*Populus deltoides*	
	Fresh wt.	Dry wt	Fresh wt.	Dry wt.
Control	*2.45* ±0.27	*0.174* ±0.06	*2.886* ±0.58	*0.253* ±0.13
+ve 2 μA	1.98 ±0.62	0.167 ±0.13	2.54 ±0.43	0.193 ±0.10
+ve 1 μA	2.74 ±0.29	0.189 ±0.04	2.78 ±0.28	0.180 ±0.09
-ve 2 μA	2.50 ±0.46	0.188 ±0.03	2.89 ±0.46	0.250 ±0.18
-ve 1 μA	2.41 ±0.25	0.173 ±0.11	2.63 ±0.64	0.287 ±0.07

Table 4.6 Electrical Stimulation of Shoot formation in *Nicotiana tabacum* at different intervals of days after inoculation in shooting medium (average of 25 replicates)

Treatments	Increase in No. of Shoots Days after inoculation 20	25	30	35	40	45
Control	0	0.25 ±0.08	3.69 ±0.37	6.08 ±1.05	6.08 ±1.28	6.08 ±0.93
+ve 2 μA	1.93 ±0.85	4.62 ±0.74	8.17 ±1.26	12.48 ±3.51	16.77 ±2.94	19.15 ±3.20
+ve 1 μA	4.13 ±1.17	9.79 ±2.31	18.27 ±2.70	22.15 ±4.28	22.81 ±3.94	23.28 ±3.25
-ve 2 μA	4.15 ±0.93	22.60 ±4.53	27.72 ±4.15	27.68 ±4.67	32.84 ±2.83	32.86 ±4.90
-ve 1 μA	1.85 ±0.69	12.62 ± 2.17	34.08 ±5.83	41.73 ±5.13	42.86 ± 3.72	48.28 ±2.71

Table 4.7 Electrical Stimulation of Shoot formation in *Populus deltoides* at different intervals of days after inoculation in shooting medium (average of 25 replicates)

Treatments	Increase in No. of Shoots Days after inoculation 20	25	30	35	40	45
Control	0	0	6.49 ±0.26	12.28 ±1.63	17.84 ±1.51	20.36 ±2.10
+ve 2 μA	1.18 ±0.24	10.83 ±1.72	32.57 ±1.93	54.80 ±2.67	72.65 5.37	74.16 3.82
+ve 1 μA	1.25 ±0.32	8.47 ±1.19	26.60 ±1.38	51.38 ±2.73	67.26 ±7.21	69.51 ±4.29
-ve 2 μA	1.27 ±0.19	22.60 ±4.68	51.32 ±2.36	97.64 ±7.15	113.26 ±7.65	116.79 ±10.52
-ve 1 μA	2.15 ±0.41	23.24 ±4.30	48.39 ±5.72	105.29 ±4.84	128.48 ±0.55	131.27 ±9.86

Fig.-4.1 Effect of Passing Electric Current on *Nicotiana tabacum* in presence of IAA

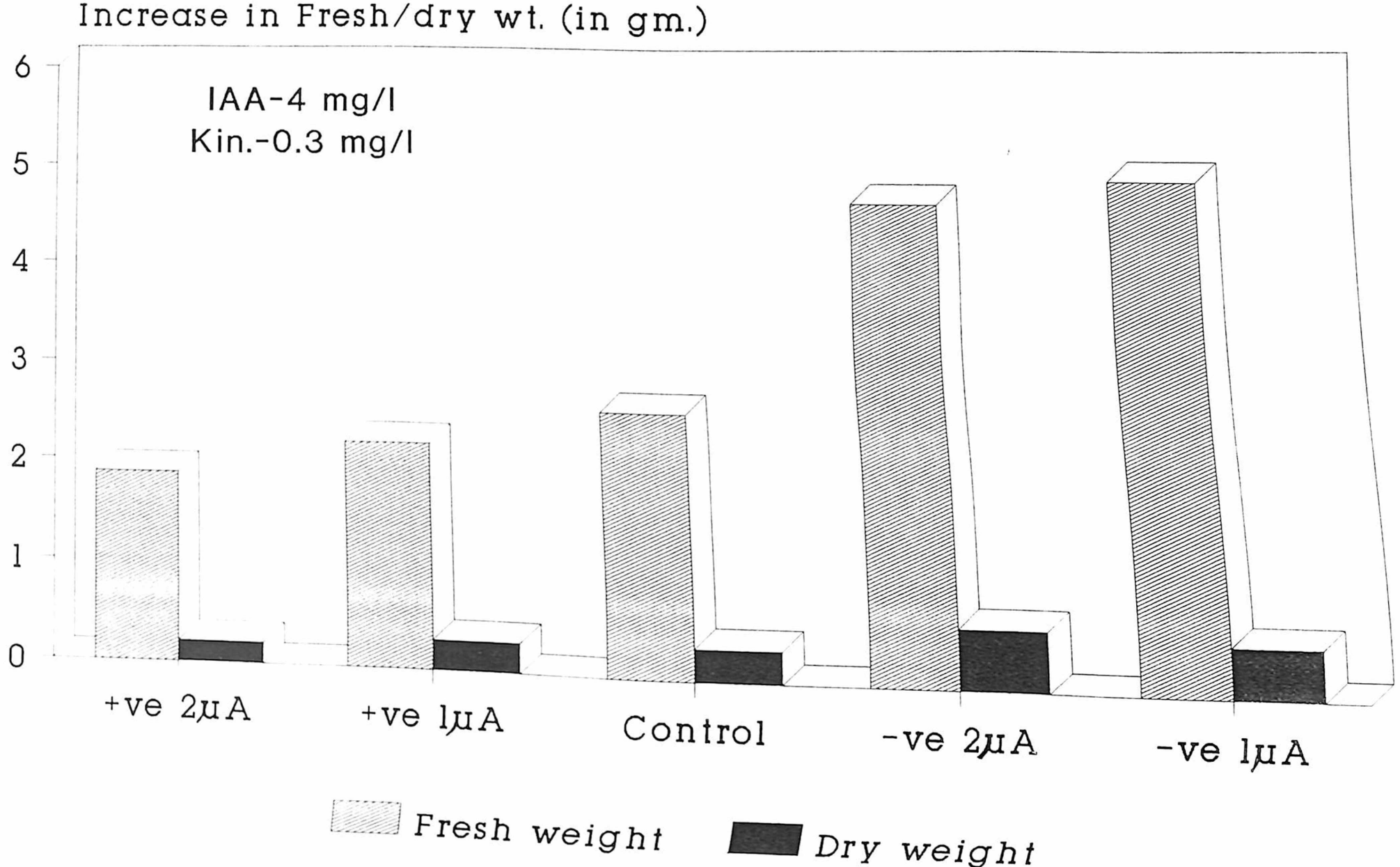


Fig.-4.2 Effect of Passing Electric Current on *Nicotiana tabacum* In presence of IAA

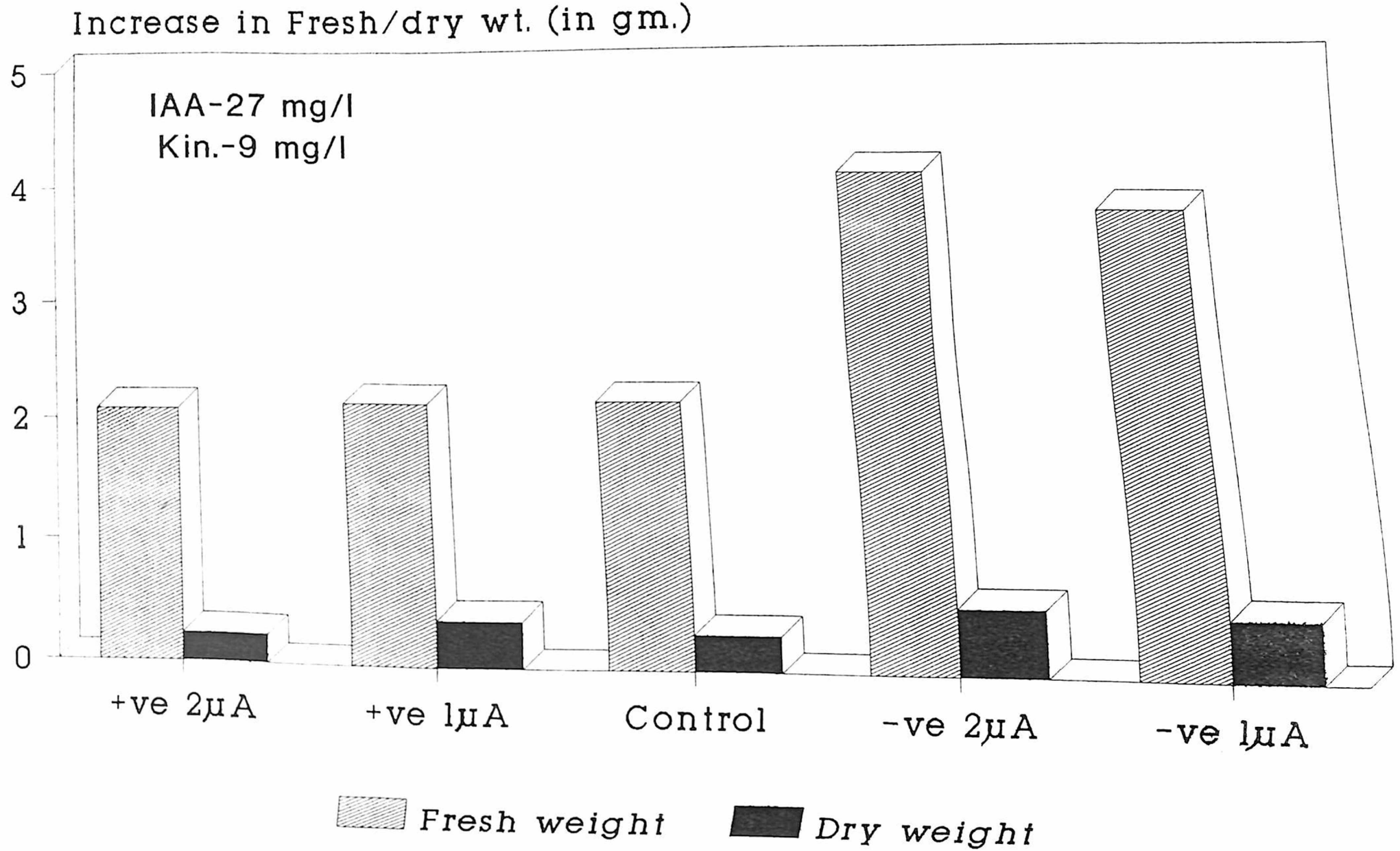


Fig. 4.3 Effect of Passing Electric Current on *Nicotiana tabacum* In Absence of IAA

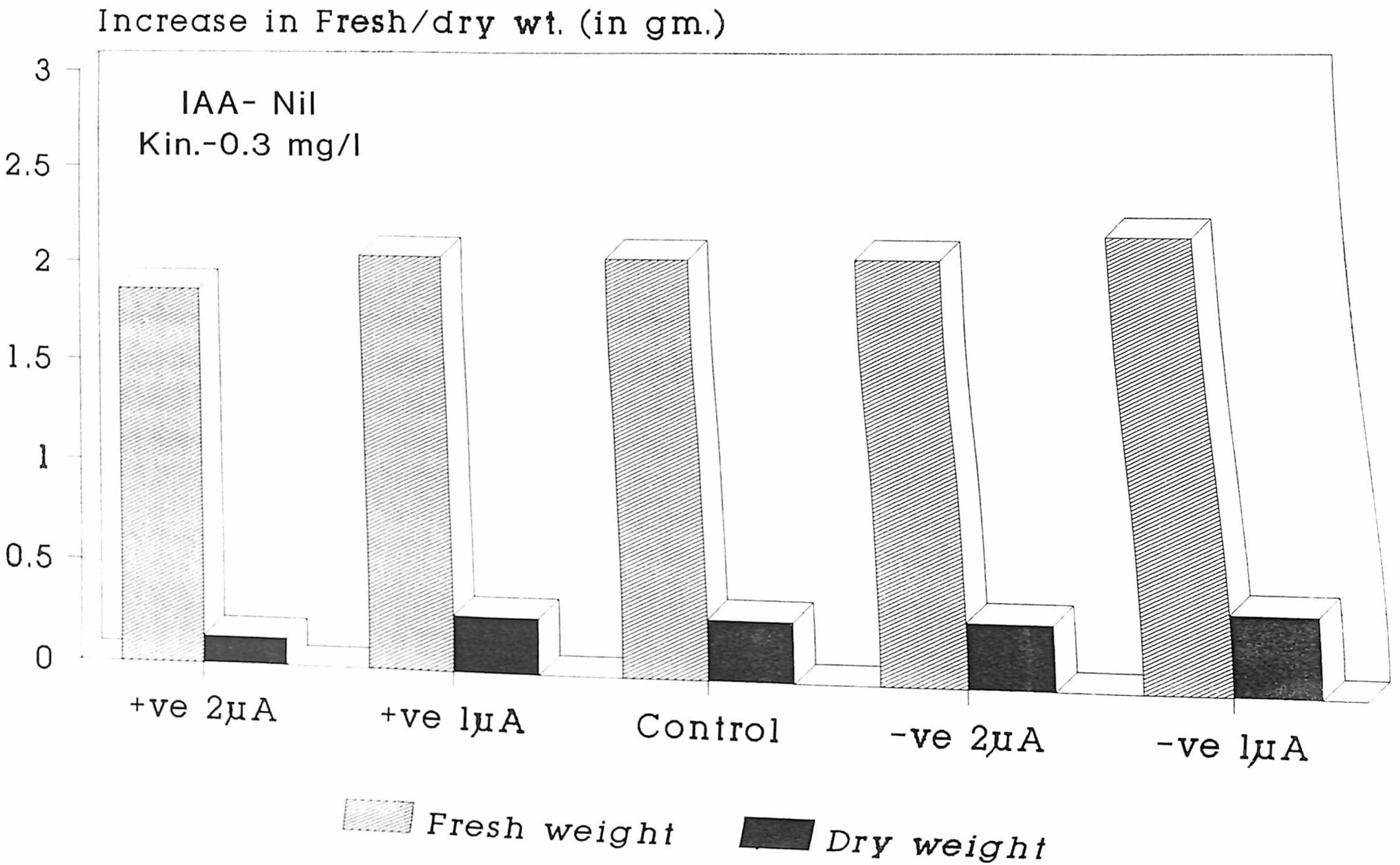


Fig.-4.4 Effect of Passing Electric Current on *Nicotiana tabacum* in presence of IAA and TIBA

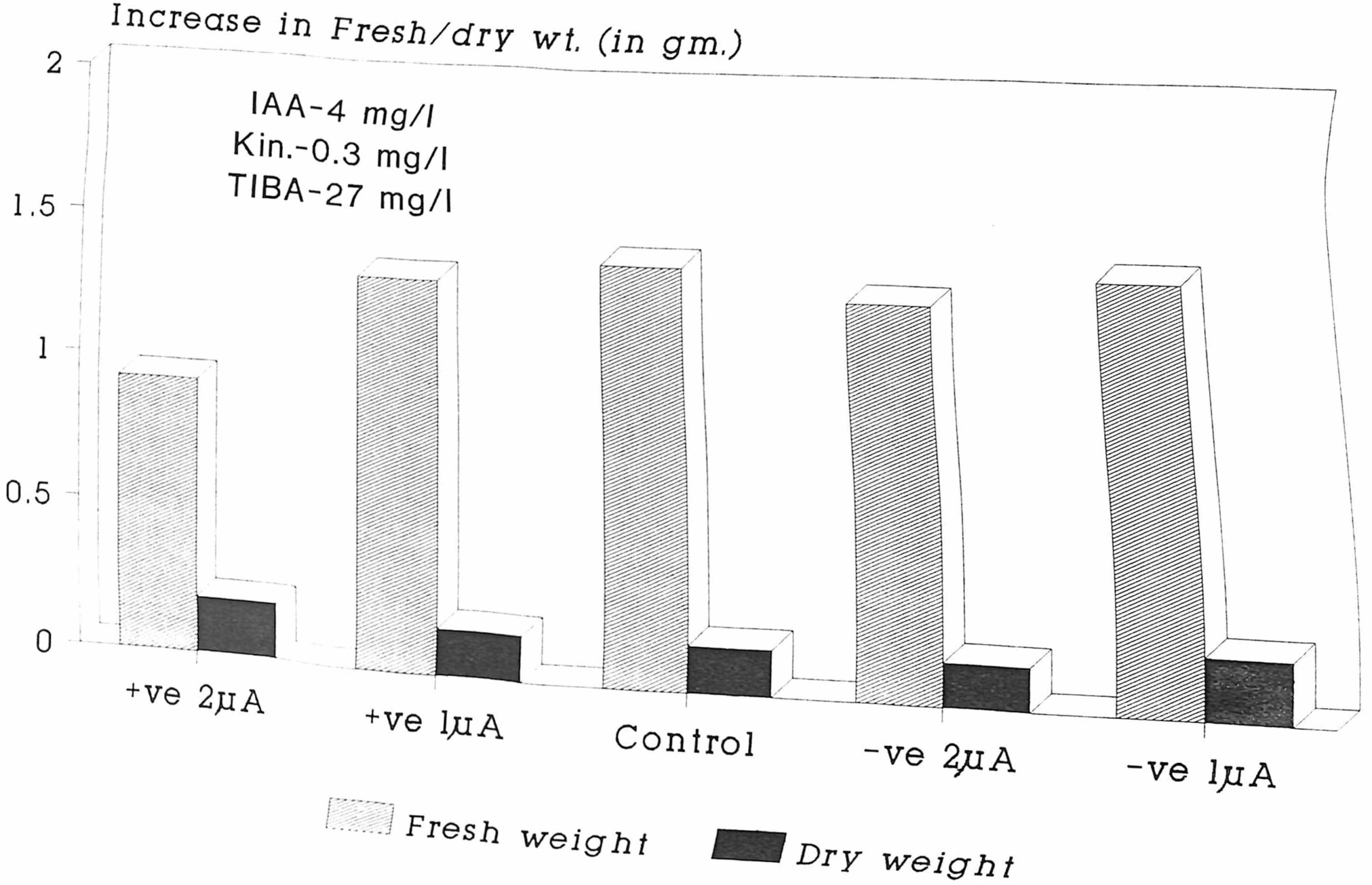


Fig.-4.5 Effect of Passing Electric Current on *Nicotiana tabacum* in presence of 2,4-D

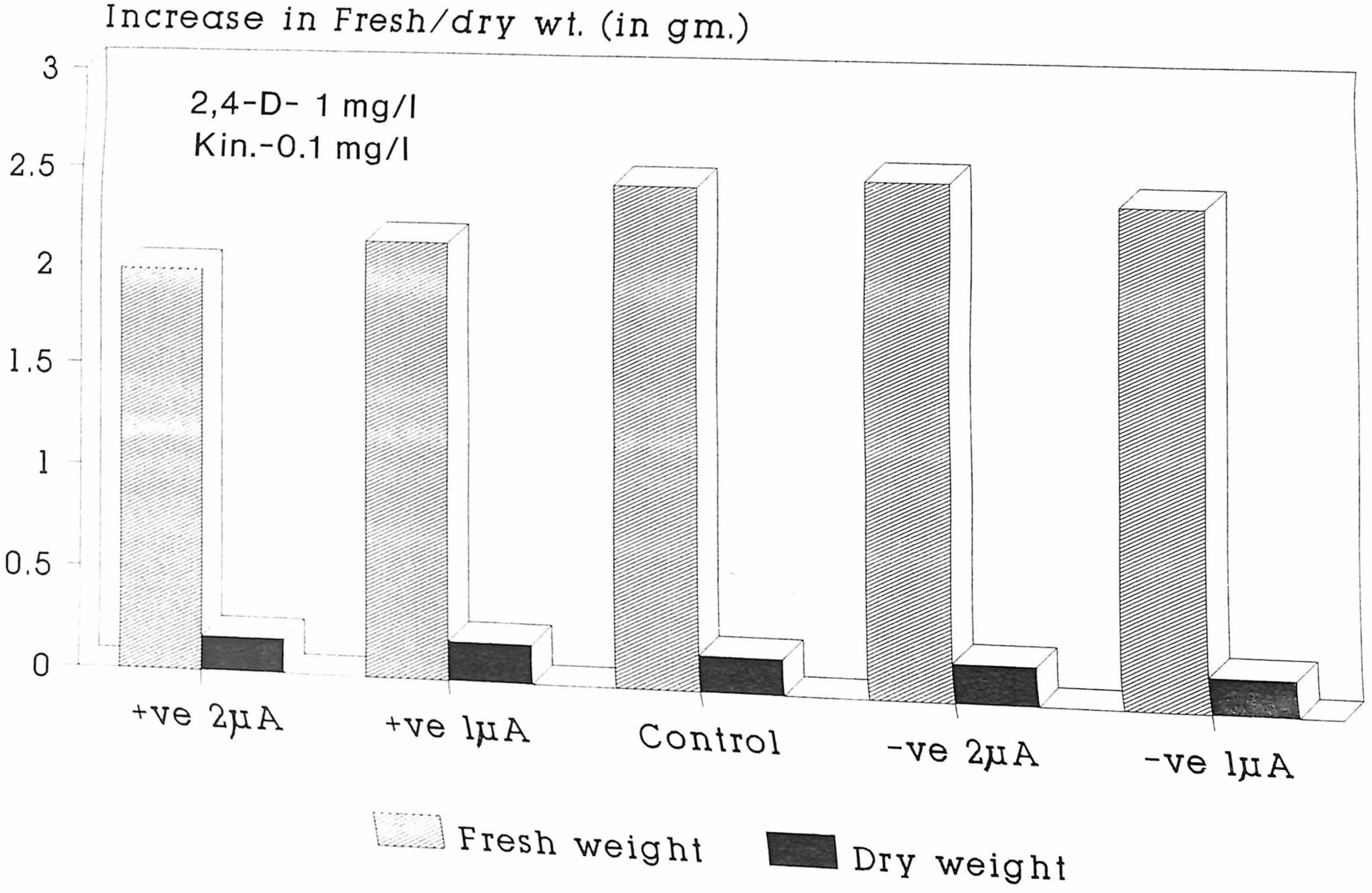


Fig.-4.6 Electrical Stimulation of total shoot formation in *Nicotiana tabacum*

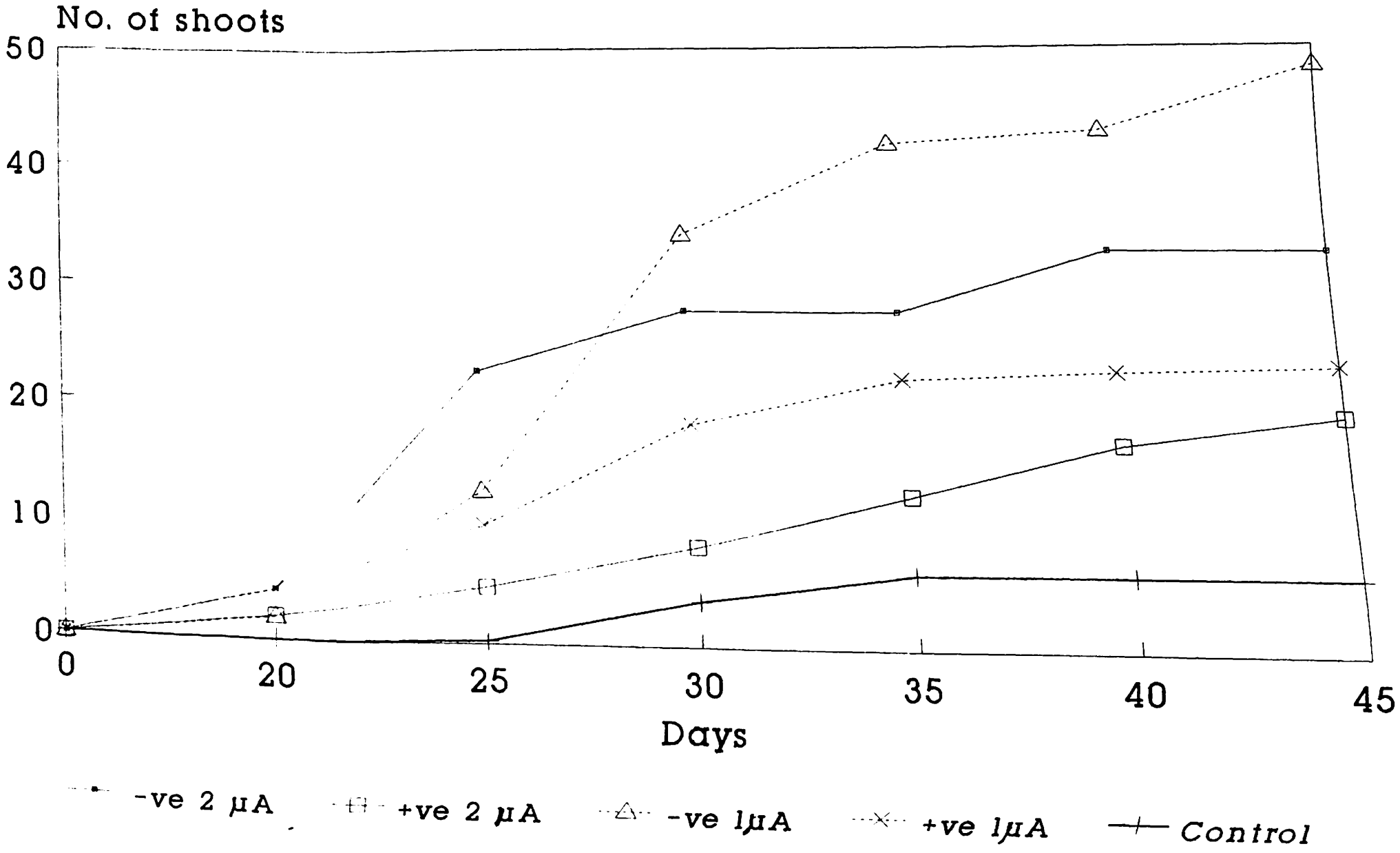


Fig.-4.7 Effect of Passing Electric Current on *Populus deltoides* in presence of IAA

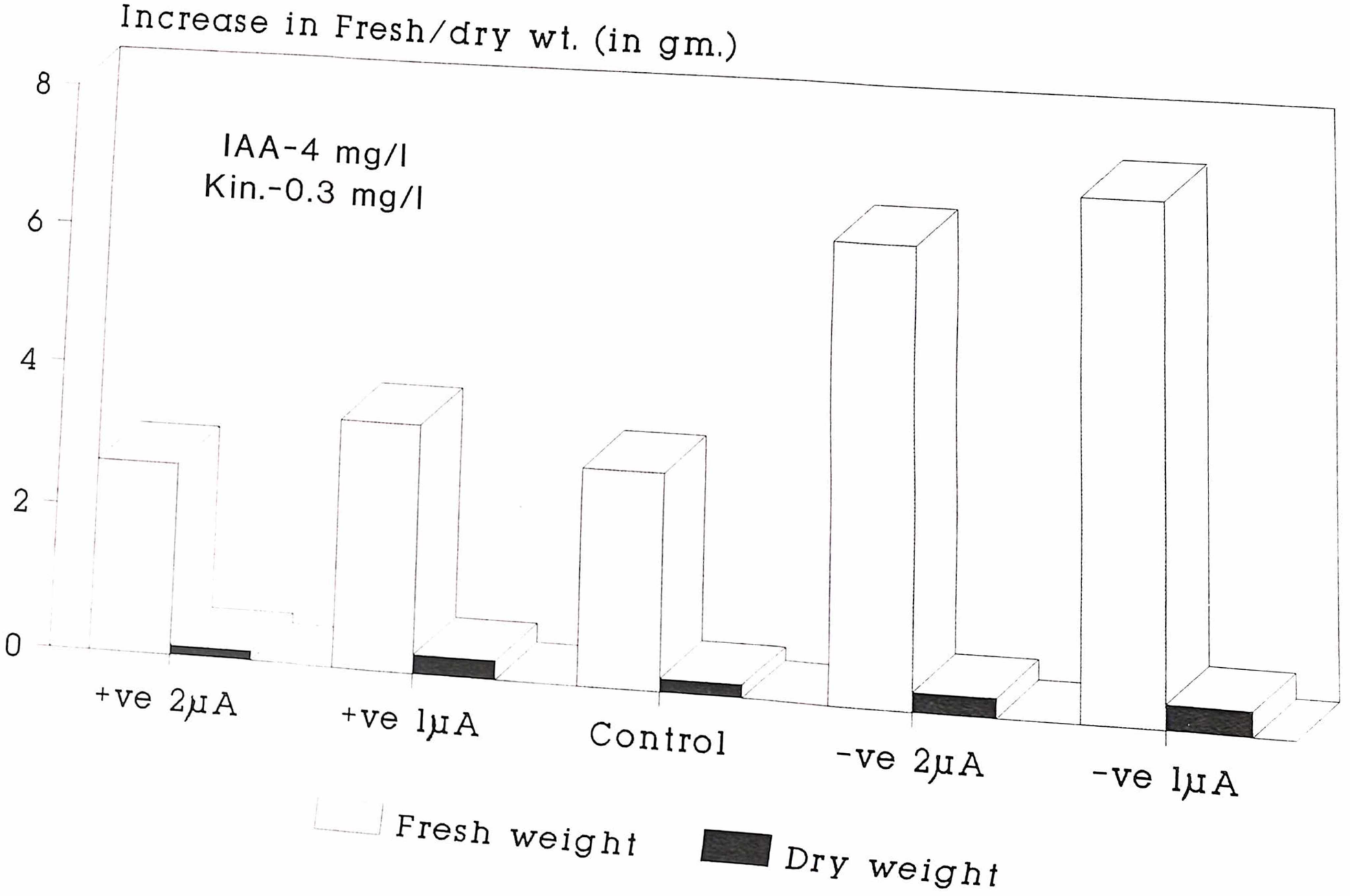


Fig.-4.8 Effect of Passing Electric Current on *Populus deltoides* in presence of IAA

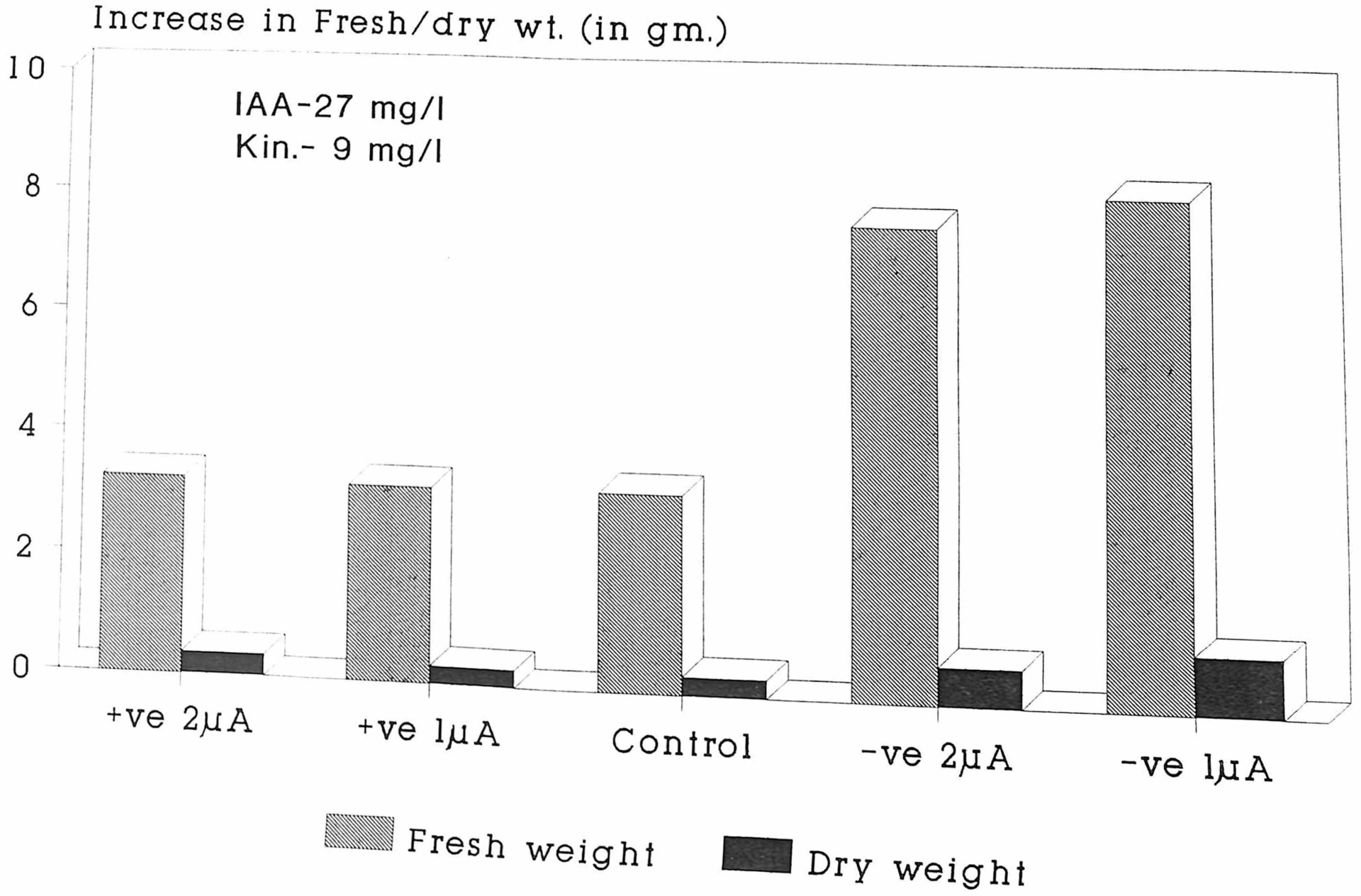


Fig.4.9- Effect of Passing Electric Current on *Populus deltoides* In Absence of IAA

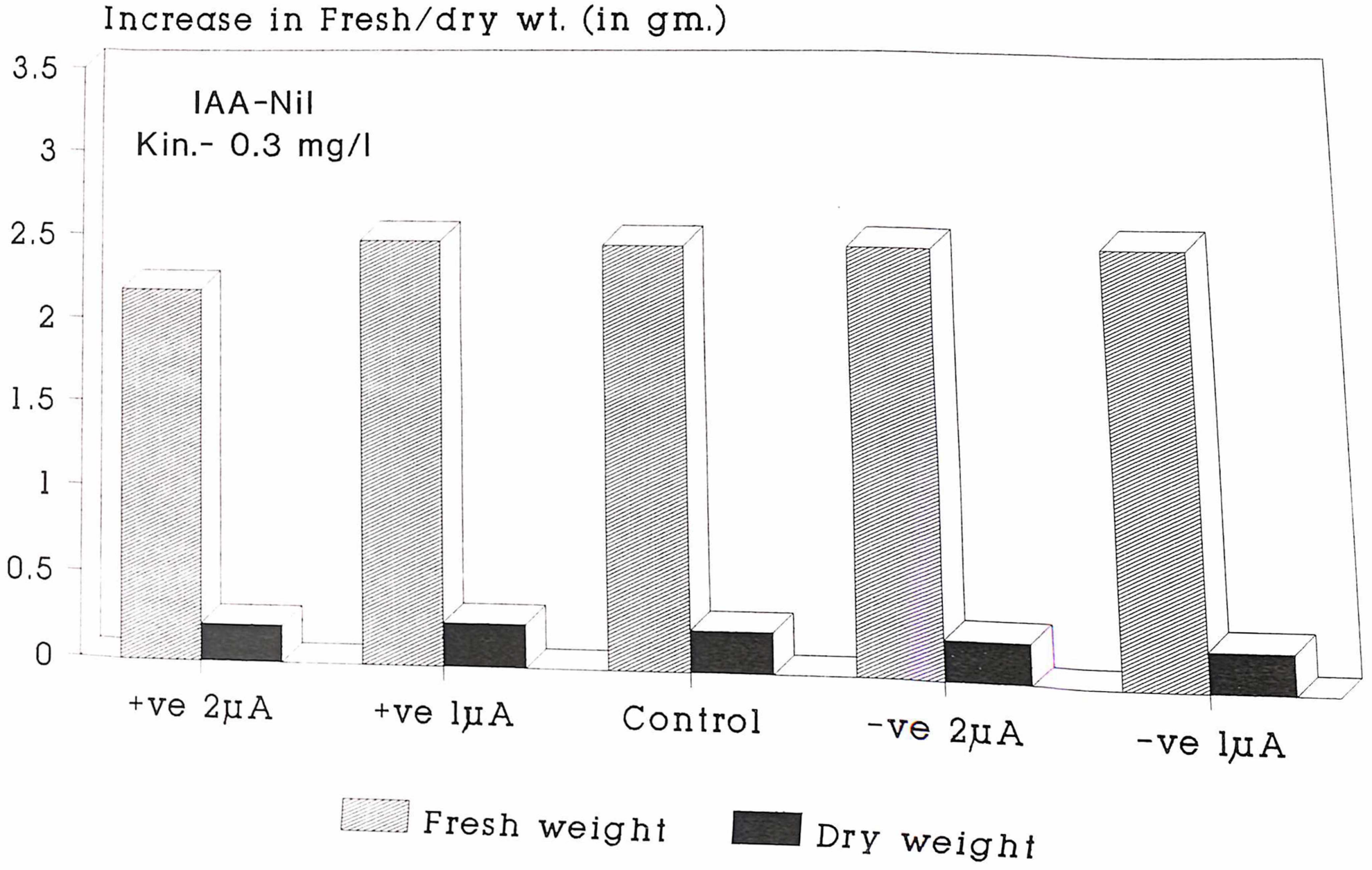


Fig.-4.10 Effect of Passing Electric Current on *Populus deltoides* In presence of IAA and TIBA

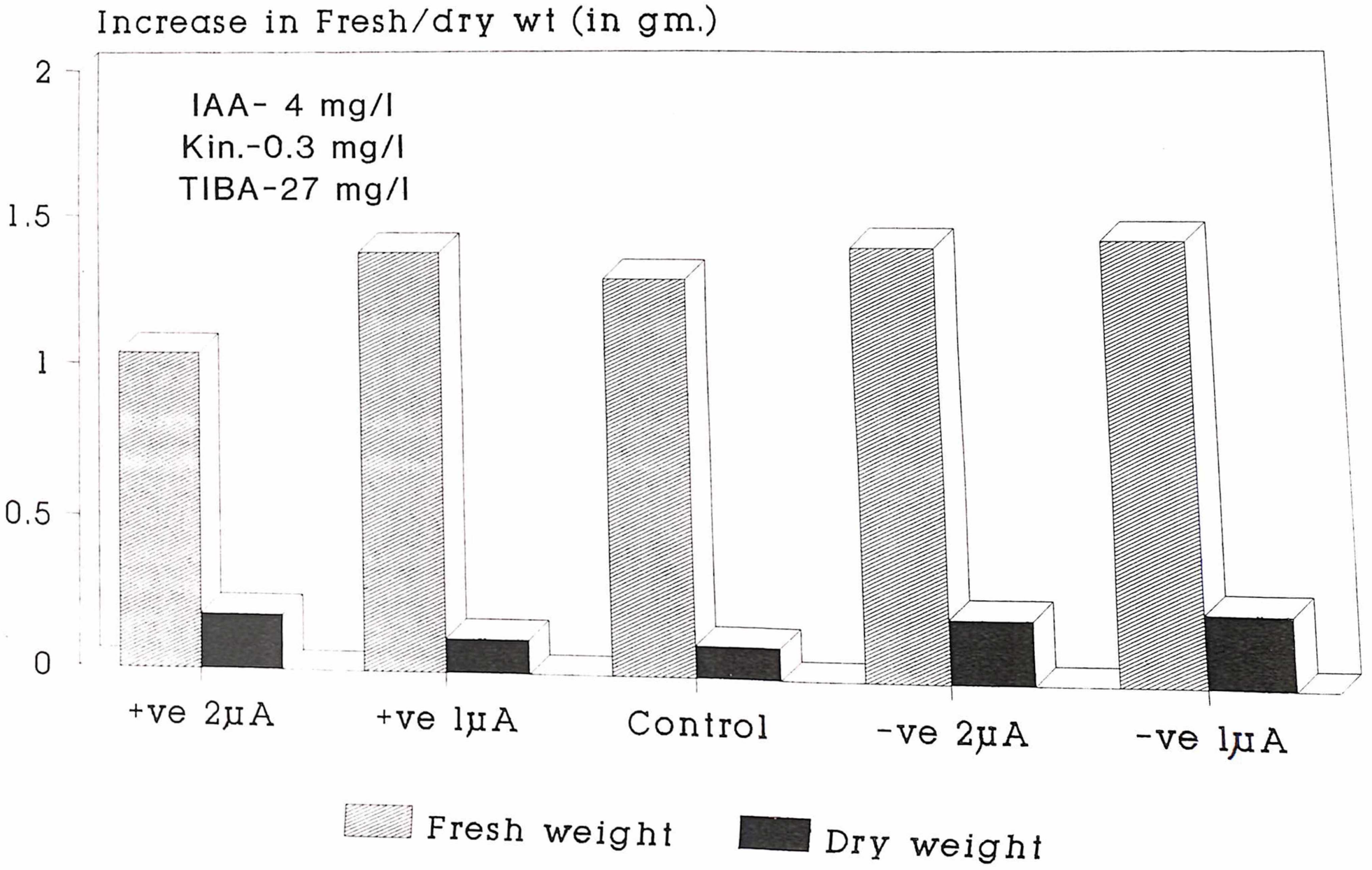


Fig.-4.11 Effect of Passing Electric Current on *Populus deltoides*

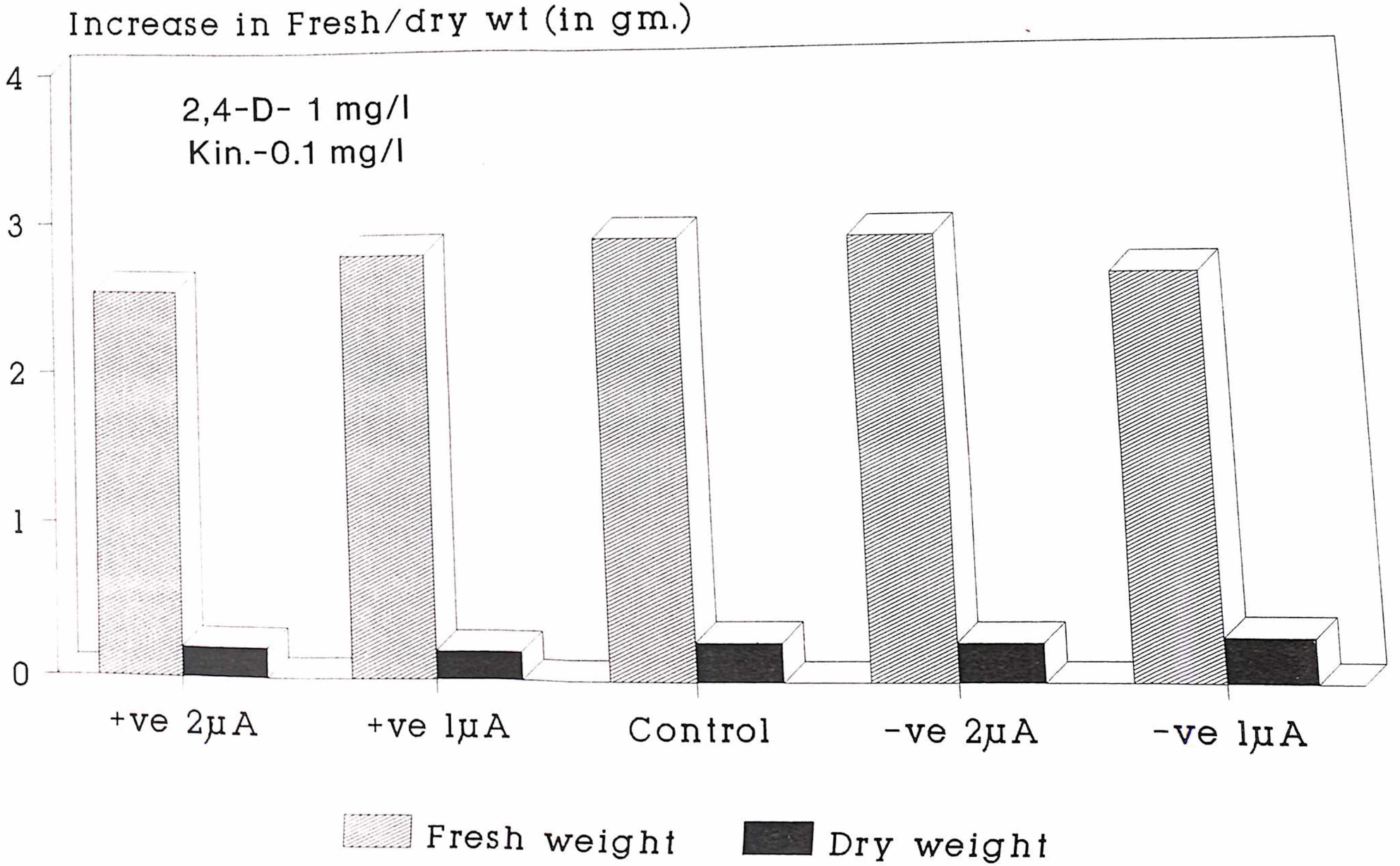


Fig.-4.12 Electrical Stimulation of total shoot formation in *Populus deltoides*

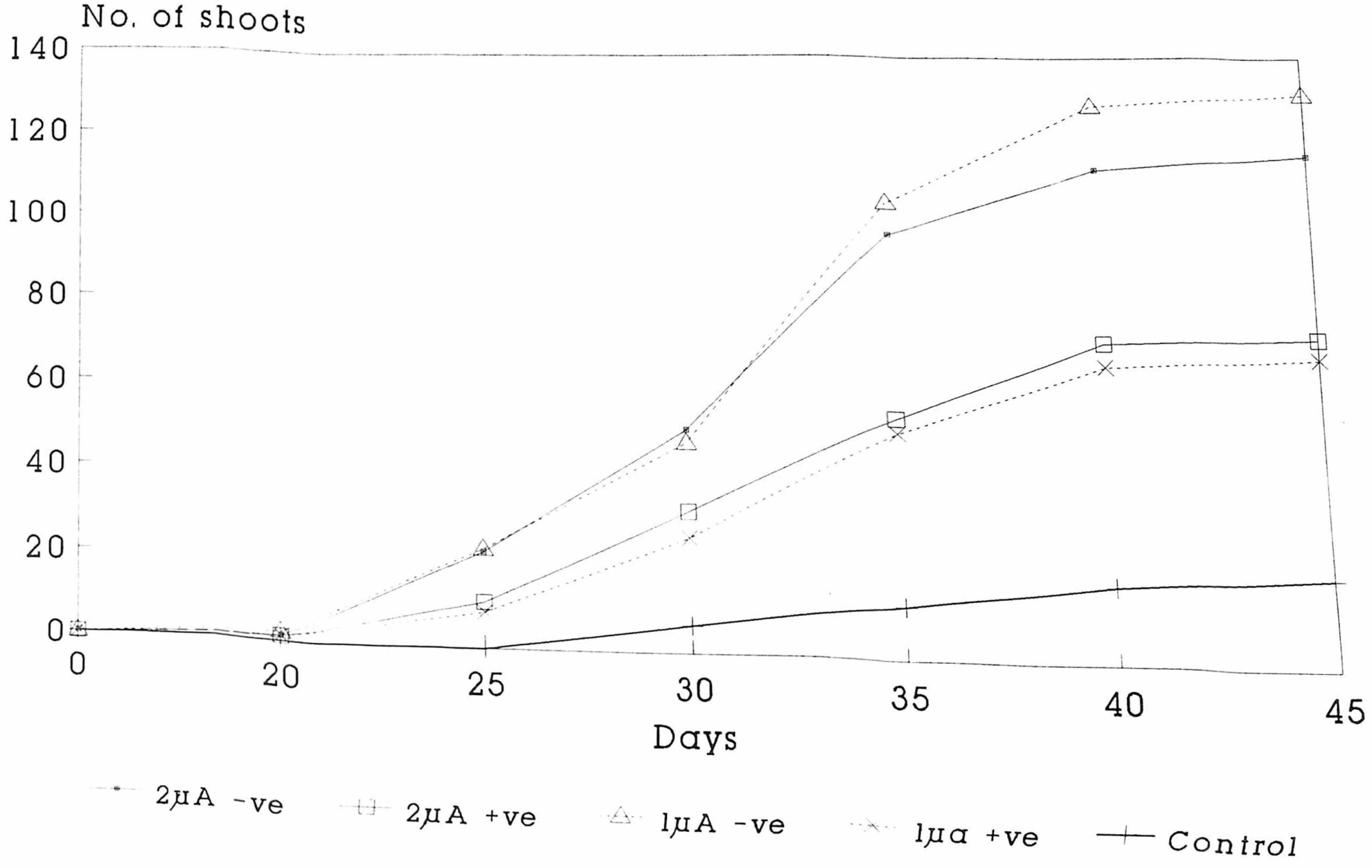


Plate 4.1: Comparison of Shoot formation (A) and undifferentiated Growth (B) in *Populus deltoides*

1. "Callus negative" treatment
2. "Callus positive" treatment
3. Control with electrodes
4. Control without electrodes

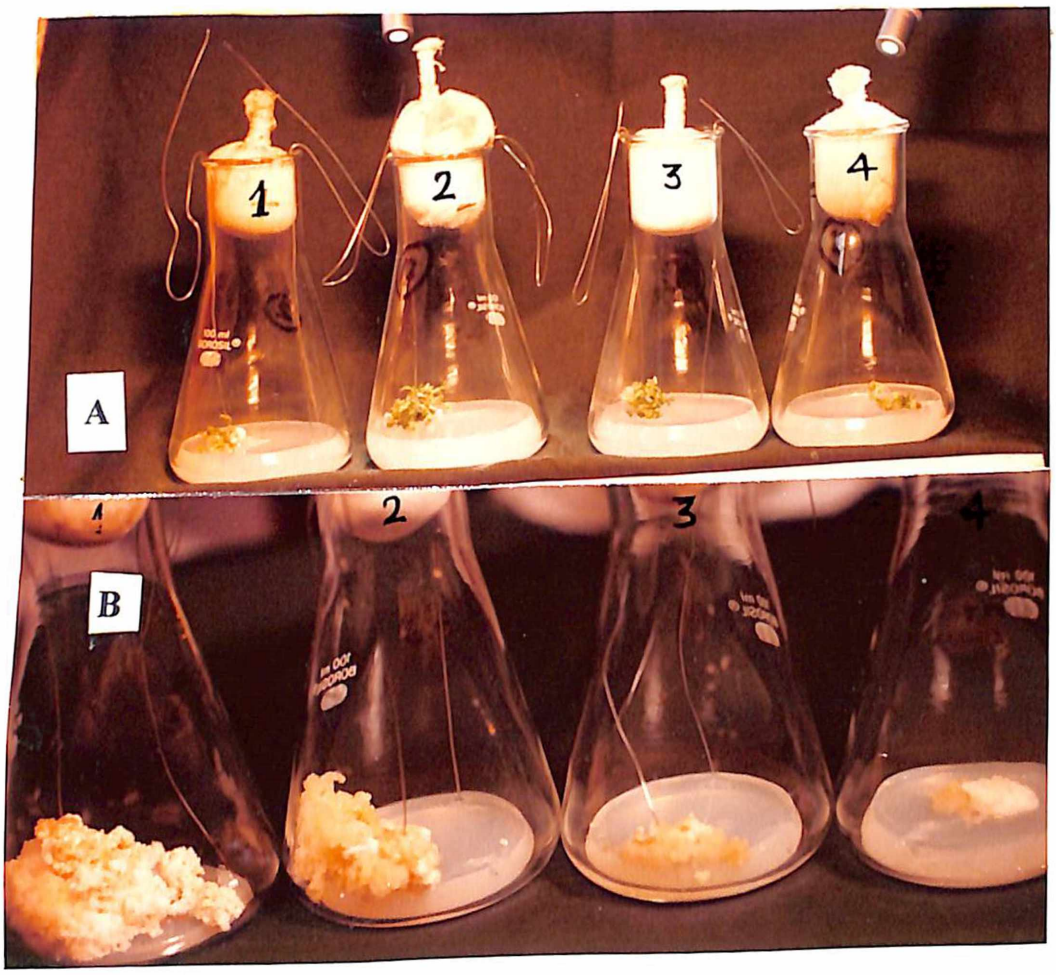

1
2
3
4
A
1
2
3
4
B

Plate 4.2: A- Regeneration in "callus negative" treatment in *Nicotiana tabacum*
B- Regeneration in "callus negative" treatment in *Populus deltoides*


A
B

Chapter 5

Electrical Control of Growth: The cytomorphological Evidence to the Phenomenon

5.1 Introduction

There are large number of reports on cellular polarity being associated with and controlled by weak electric currents flowing through cell in various unicellular and multicellular organisms (Jaffe & Nuccitelli, 1997 and references there in). Goldworthy and Rathore (1985) have reported a 70 percent stimulation of growth by applying weak electric currents in tobacco callus cultures. Dutta (1992,1993) observed the similar phenomenon in poplar cultures. They suggested the mechanism of the said electrical stimulation being polar uptake of auxin i.e. IAA. On the basis of the previous studies by Braton and Henery (1977) and Morris (1980) on the intact plants, Medvedev and Markova (1990) with the help of radio labeled IAA proved polarized uptake of IAA in *Zea mays* coleoptile by electrical influence. In the present chapter, results of independent studies have been carried out on the percentage increase in growth parameters viz. weight, cell volume and number and also changes in cell shape, under the influence of electric current of 1 μA.

5.2 Materials & Methods

The experiments (as described in chapter 4) were repeated to raise callus cultures of *Nicotiana tabaccum* and *Populus deltoides*, and the electric current of the order of 1.0 μA and 2.0 μA were passed through these cultures. Since the growth rate was found to be less luxuriant in case

of 2.0 μA current level, only results for 1.0 μA have been recorded. The reproducible results were observed for both *Nicotiana tabaccum* and *Populus deltoides*. The increase in callus growth was adjudged on the basis of the following parameters:

(a) Increased rate of division expressed as cell number

(b) Swelling of cells i.e. accumulation of water inside cells in terms of dry and fresh weight ratio and/or

(c) Elongation of cells i.e. cell shape index.

The cell number (per gm. fresh weight) i.e. packed cell volume (PCV) and cell shape index (isodiameteric and elongated cells ratio), and the cell volumes were calculated under a microscope using heamocytometer and occulometer. Prior to examination, the callus tissues were macerated overnight in 1% chromium trioxide solution. The cell volume (V) for isodiameteric (i.e. circular) and elongated cells were calculated by using following solid geometry formulae:

a) Isodiameteric cells:

$$V = 4/3\pi R^3$$

Where R is cell radius

b) Elongated cells:

L is cell length

$$V = 2\pi R^2 L$$

π is 3.14

5.3 Results

At both 1.0 μA and 2.0 μA current levels there was a significant increase in fresh weight in the callus negative treatment, while the callus positive and control treatments, do not show much difference (Table 4.1 and, Fig. 4.1 and 4.7). The 1.0 μA current level shows much luxuriant growth as compared to 2.0 μA. Thus the results has been recorded only for 1.0 μA. The initial dry weight and fresh weight ratio was found to be 10.4 and remained unchanged throughout the experiment and various treatments. This suggests that electrical influence do not alter the water retention capacity of the cells and only the biomass increases. In other words the increase in biomass is not due to swelling of cells.

The experiment reveals that the cell numbers per gm. fresh weight or the packed cell volume (PCV) and cell shape were altered by virtue of electrical influence. The cytomorphological studies reveals that the current influences the number of cells per gram fresh tissue and cell size and shape. The PCV was increased from 4.4 $x10^{6}$ cells in 'callus positive' to 8.29 $x10^{6}$ cells in 'callus negative' polarities (Table 5.1, 5.2 and 5.3 and, Fig.5.1, 5.2, 5.3, 5.4, 5.5 and 5.6). The PCV of control experiments remained similar to the 'callus positive' polarity (Table 5.1 and Fig.5.1 and 5.4). This may be due to the increased cell division and cell enlargement.

The tendency of cells to elongate increased progressively under the influence of electric current. The results on the percent increase in the number of cell in various volume ranges are recorded in *Nicotiana tabacum* (Table 5.2) and *Populus deltoides* (Table 5.3). The elongated

cells were found to be 8% at 'callus positive' polarity, but they increased to 22.9% when callus was made negative polarity. It was also seen that of the 22.9% elongated cells at 'callus negative' polarity only 5.76% were found in the maximum volume regime of $>500x10^{-15}$ m^3 and 0.96% of isodiameteric cells was found in the maximum volume regime of 301-500 $x10^{-15}$ m^3. On the contrary, the maximum volume range for elongated and isodiameteric cells were found to be in 101-200$x10^{-15}$ m^3 and 51-150$x10^{-15}$ m^3 range respectively in 'callus positive' polarity. The same was found to be more or less true for control callus. These results reveal that the PCV and cell volume becomes doubled in 'callus negative' polarity as compared to the 'callus positive' and control cultures.

5.4 Discussion

McLeod (1992) suggested that the cell profile is a critical parameter defining the cellular sensitivity to extracellular fields. The present investigation has revealed that the electric current altered the cellular morphology and volume favouring cellular elongation from isodiameteric condition in 'callus negative' experiments. This may possibly be induced by the polarized flow of IAA in callus cells under the exogenous applied current. The polarized flow of IAA from root to shoot to leaves under the influence of electric field in whole plant has been demonstrated in several species (Braton and Henery, 1977 & Morris, 1980). Earlier Webster & Schrank (1953) in their comperative study on various growth hormones, found that IAA was strongly polarizable and NAA was only weakly polarizable among the various auxins. It is also well established that auxins promotes cell elongation (Audus, 1972). According to Evans (1974) auxins stimulates the elongation of coleoptile and stem cells within 10-20 minutes of application. Stimulation of cell elongation by auxin in hypocotyl, epicotyl and stem cells has

also been reported in number of dicots (Cleland et al., 1968; Bookly et al. 1970; Kazama & Katsumi, 1974; etc.).

The observations that electrical current changed the cell volume without altering the water retention capability of cells was evident from the unchanged dry to fresh weight ratio in the electrically treated cultures. Studies on the influence of electrical current on the protoplast of several species have demonstrated stimulation of cell wall regeneration, thereby tissue growth (Rech et al., 1987; Gupta et al., 1988).This may possibly be due to the polarized IAA flow. Enhanced metabolic biosynthesis has also been reported earlier for cell wall component biosynthesis (Evans, 1974; Rech et al., 1987; Gupta et al., 1988). The increased growth rate does require enhanced DNA and protein synthesis as suggested by the earlier workers (Rech et al., 1987; Joerbo & Brunsetedt, 1990). Considering the evidences form the studies on the growth parameters it can be concluded that under the influence of exogenous electric current even the tissues with unpolarized growth behaved like whole plants with polarized growth due to unidirectional auxin flow.

Table 5.1 Effect of Passing 1μ Current on Packed Cell Volume and Cell Shape in *Nicotiana tabacum* and *Populus deltoides* callus after 30 days of inoculation
(average of 25 replicates)

	Nicotiana tabacum		*Populus deltoides*	
Treatments	Cell Numbers per gm. Fresh wt. (10^6)	Elongated Cells (in %)	Cell Number per gm. Fresh wt. (10^6)	Elongated Cells (in%)
Control	4.34 ±1.42	7.5 ±0.36	11.54 ±1.92	9.14 ±1.24
+ve 1 μA	4.4 ±0.97	7.6 ±0.51	11.68 ±1.17	9.21 ±0.86
-ve 1 μA	8.29 ±0.65	22.9 ±2.18	23.72 ± 3.06	26.07 ±2.59

Table 5.2 Effect of Passing 1μ Current on Distribution of Circular and elongated Cells in different Volume Ranges in *Nicotiana tabacum* after 30 days of inoculation
(average of 25 replicates)

	% Number of cells in specified volume ranges									
	Volume Ranges (10^{-15})									
	Circular Cells				Elongated Cells					
Treatments	<50	51-150	151-300	301-500	<50	51-100	101-200	201-300	301-500	>500
Control	75.28	21.72	0	0	89.82	7.49	2.69	0	0	0
+ve 1 μA	86.35	13.65	0	0	87.40	9.30	3.30	0	0	0
-ve 1 μA	69.82	22..5	6.7	0.96	38.56	22.50	17.69	6.88	2.96	1.81

Table 5.3 Effect of Passing 1μ Current on Distribution of Circular and elongated Cells in different Volume Ranges in *Populus deltoides* after 30 days of inoculation
(average of 25 replicates)

	% Number of cells in specified volume ranges Volume Ranges (10^{-15})									
	Circular Cells				Elongated Cells					
Treatments	<50	51-150	151-300	301-500	<50	51-100	101-200	201-300	301-500	>500
Control	79.5	20.5	0	0	79.82	14.46	5.72	0	0	0
+ve 1 μA	65.20	34.8	0	0	78.37	18.39	3.24	0	0	0
-ve 1 μA	46.02	26.18	16.60	11.20	69.56	12.5	8.84	2.88	1.96	4.26

Fig.-5.1 Effect of 1μA Electrical Current on PCV and Cell Shape in *Nicotiana tabacum*

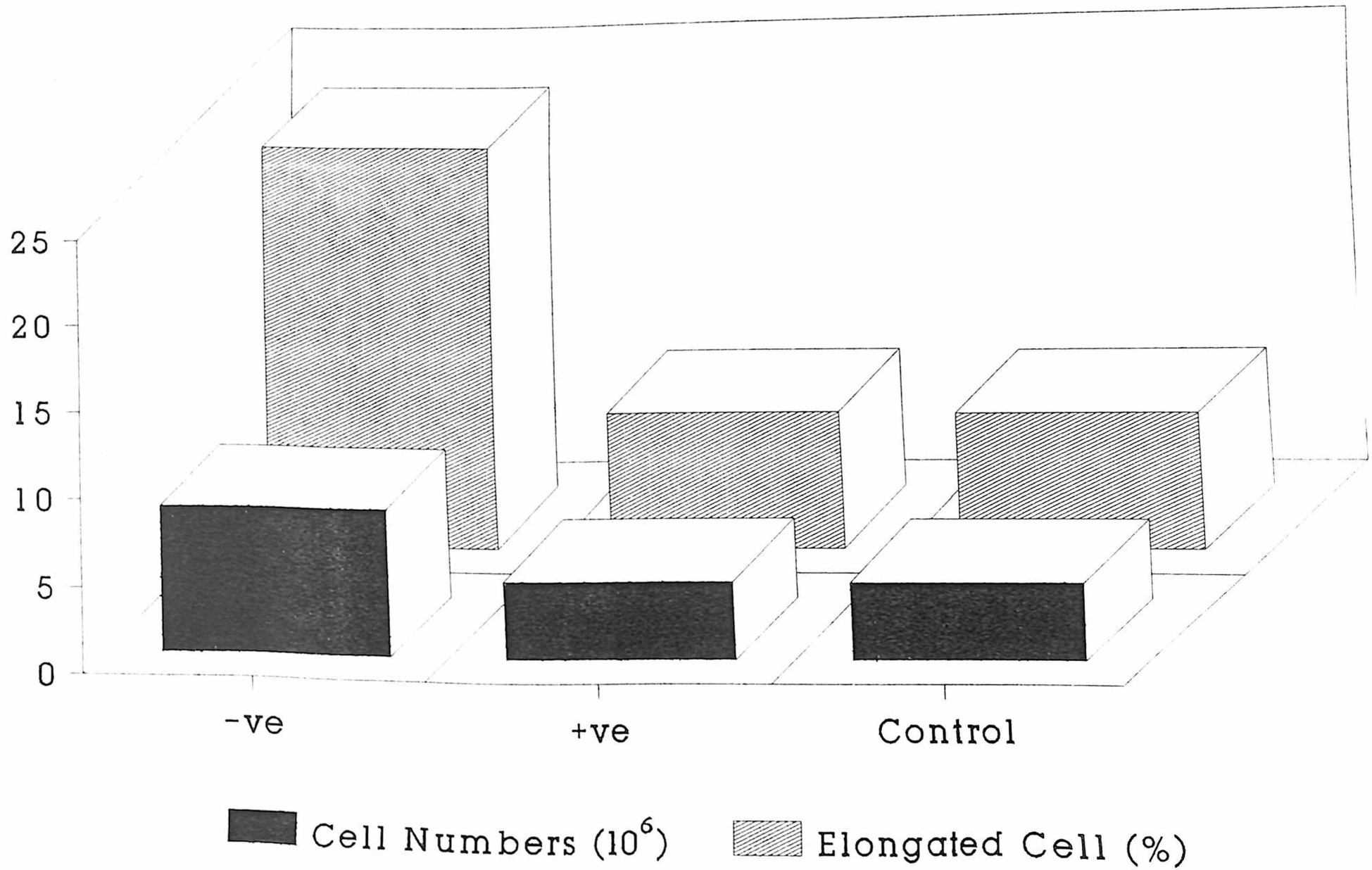


Fig.-5.2 Distribution of Circular Cells in Different Volume Ranges in *Nicotiana tabacum*

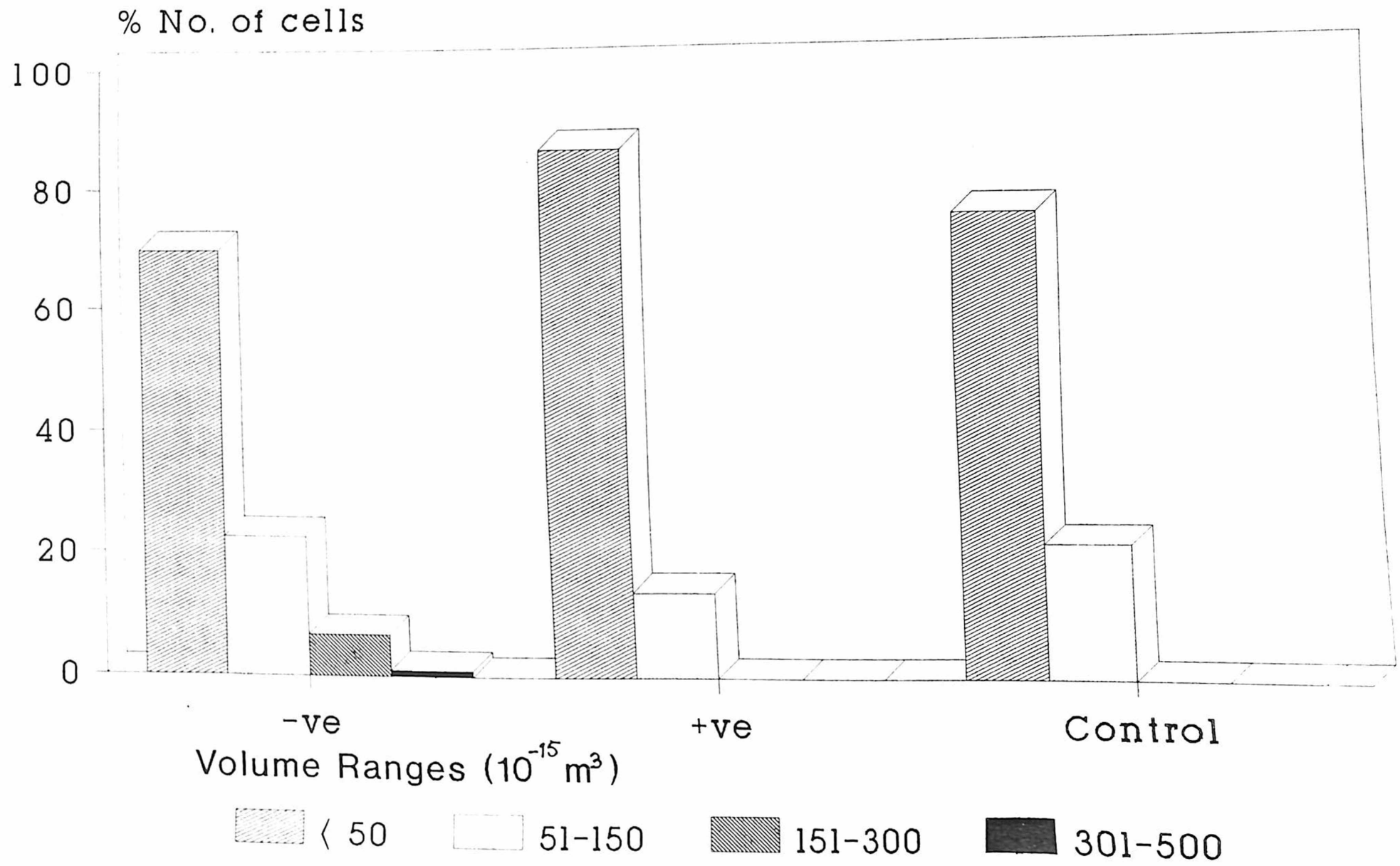


Fig.-5.3 Distribution of Elongated Cells in Different Volume Ranges in *Nicotiana tabacum*

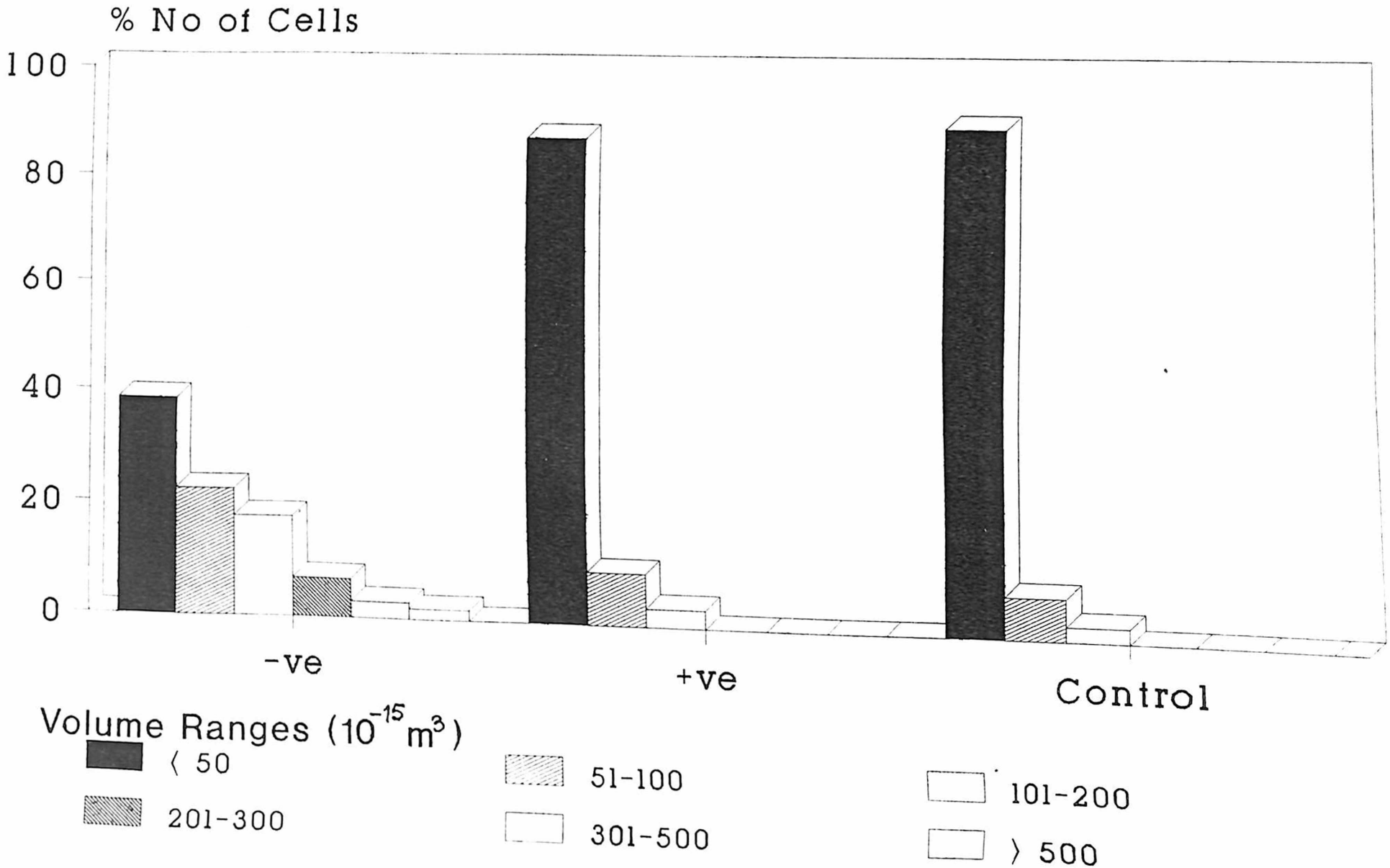


Fig.-5.4 Effect of 1μA Electrical Current on PVC and Cell Shape in *Populus deltoides*

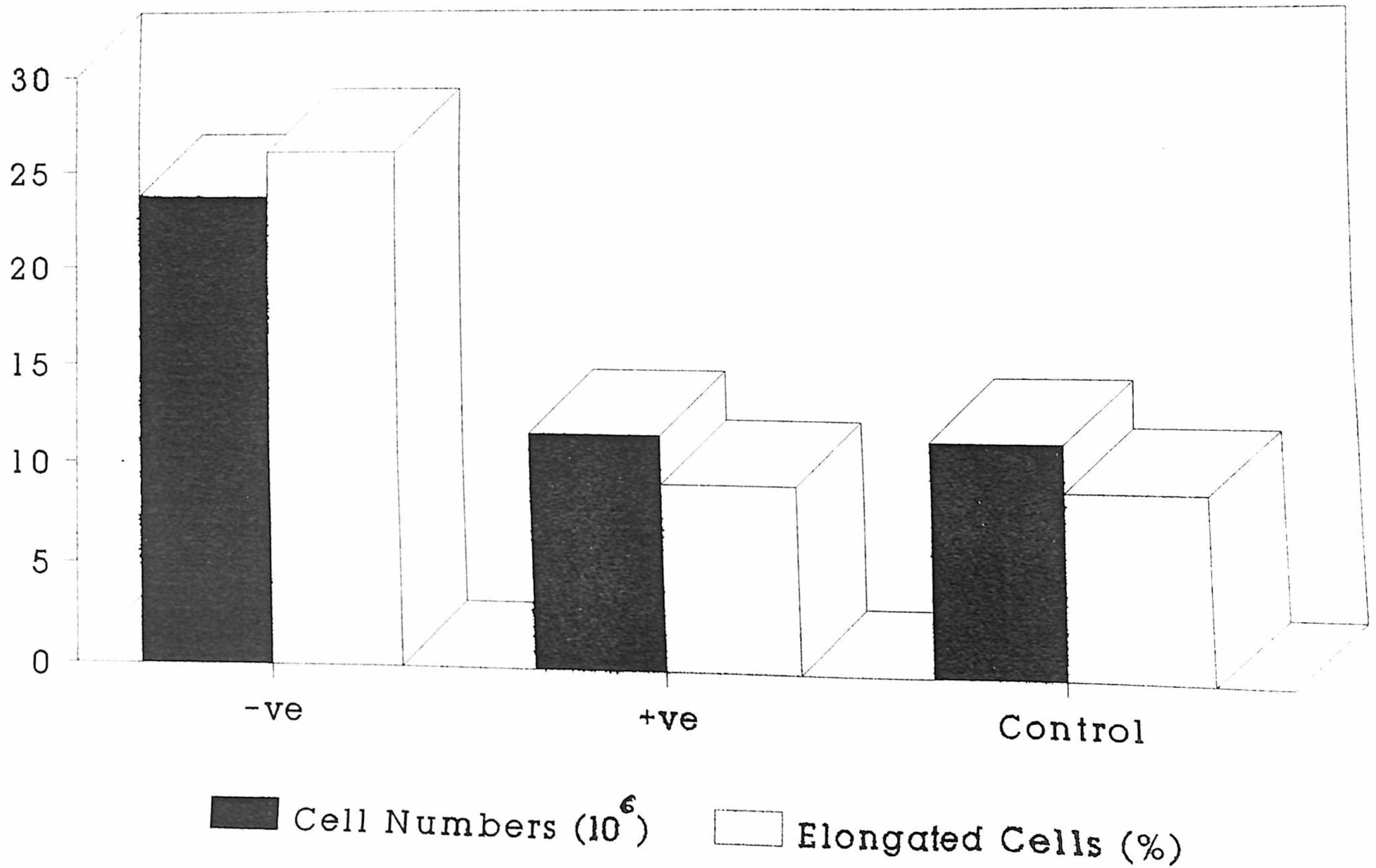


Fig.-5.5 Distribution of Circular Cells in Different Volume Ranges in *Populus deltoides*

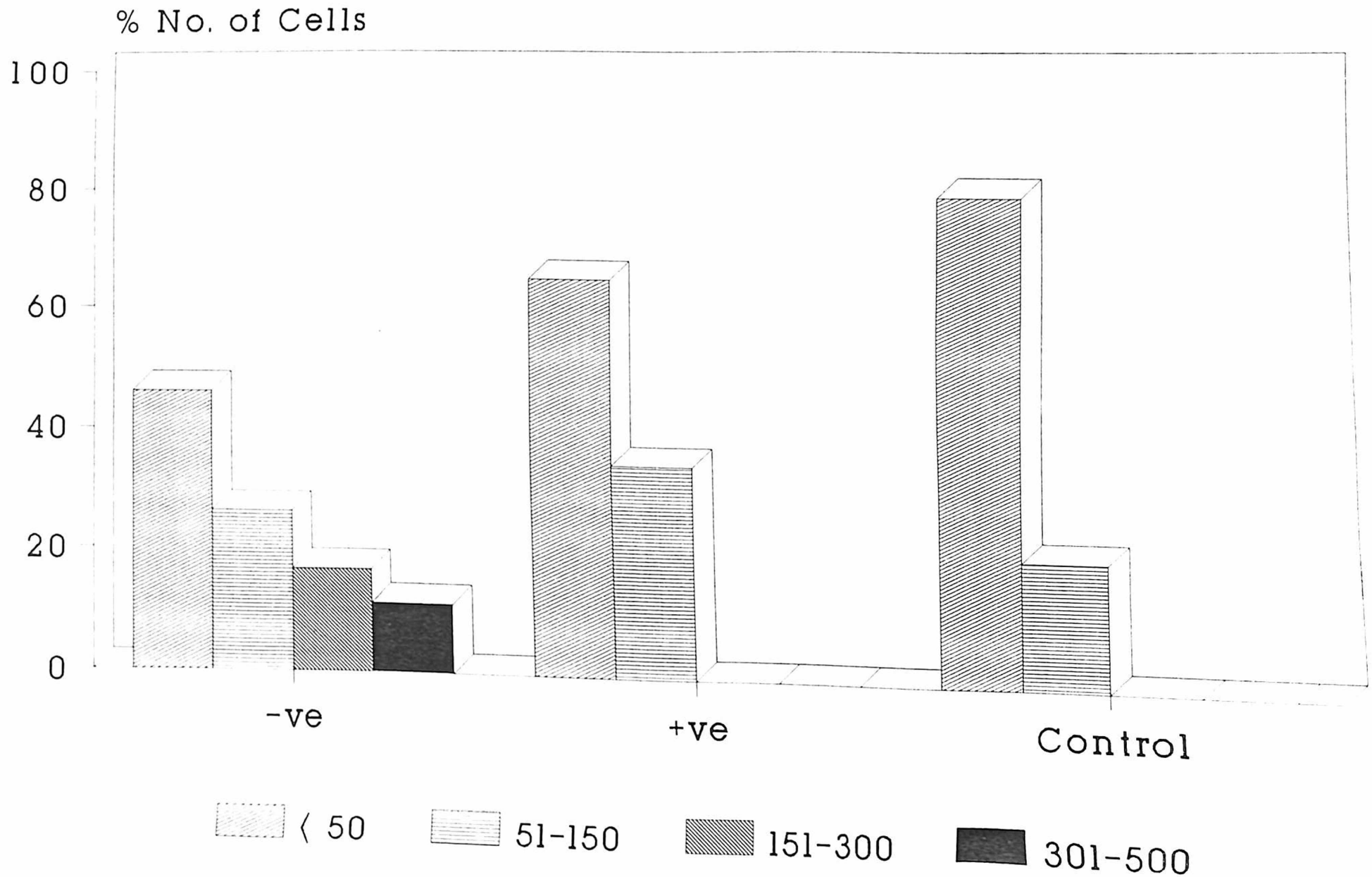
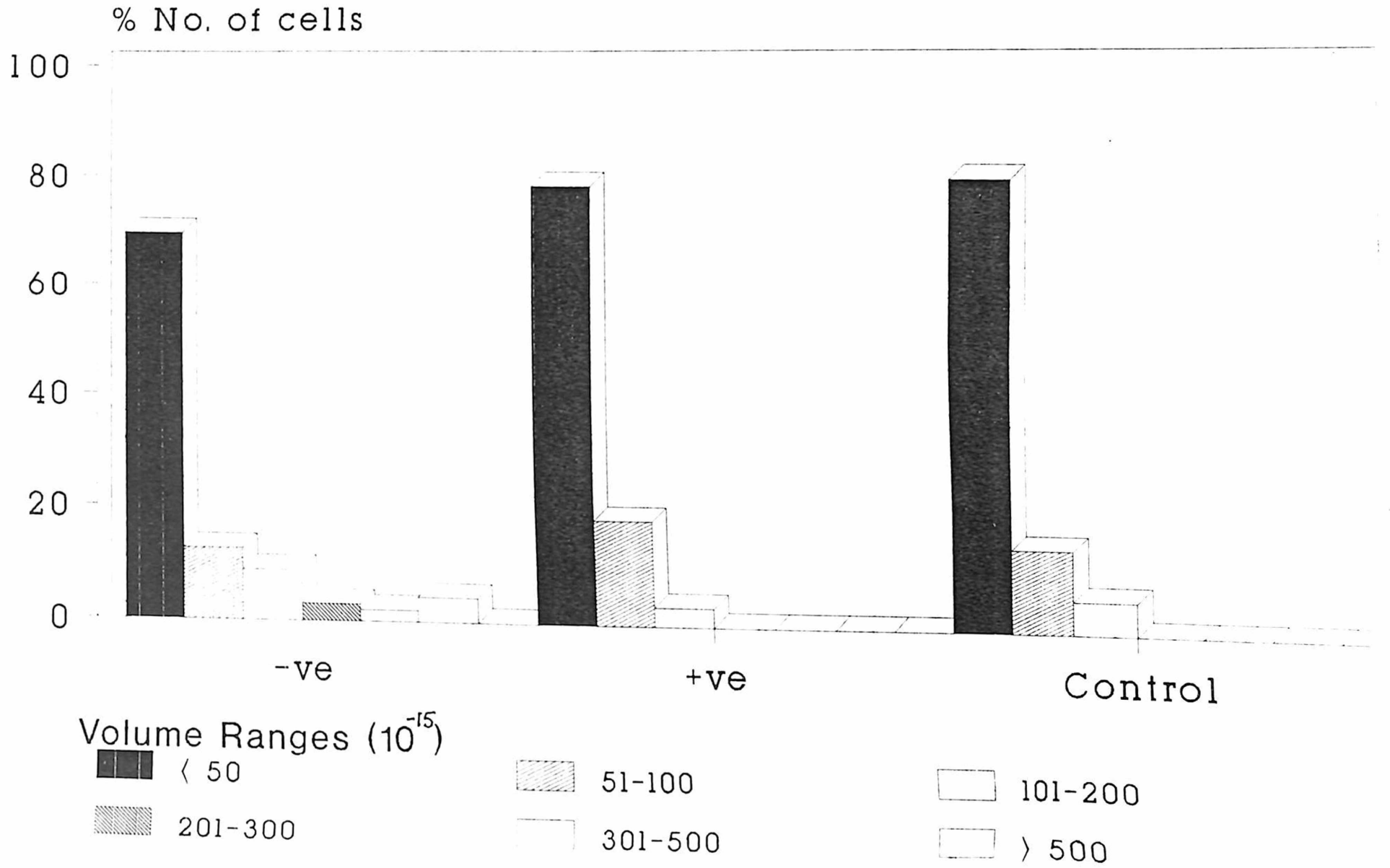


Fig.-5.6 Distribution of Elongated Cells in Different Volume Ranges in *Populus deltoides*



Chapter 6

Electrical polarization of cells by calcium ion channels

6.1 Introduction

There are many reports of externally-applied electric currents inducing polar growth in non-polar cells including the fucoid zygote (Lund, 1923), *Acetabularia* stalks with lost natural polarity (Novak and Bentrup, 1972), and moss protoplasts (Burgess and Linstead, 1982). This effect has sometimes been attributed to the forced unilateral entry of calcium (Chen and Jaffe, 1979; Robinson and Cone, 1980), but the precise mechanism is not clear.

External currents can also re-orientate the existing native electrical polarity of higher plant cells growing in tissue culture. The new polarity tends to be oriented along the flow axis of current applied (Mina and Goldsworthy, 1991). Higher current densities of the order of 100 $\mu A\ cm^{-2}$ produced the above effect within minutes, but lower densities similar to natural transcellular currents took several hours to bring such orientation. This was taken as the evidence for a mechanism by which adjacent cells in a tissue co-ordinate their polarities to support organized growth. In the present work, the experiments were carried out to provide evidence that calcium plays an important role in the polarization

process. For this purpose attempts were made to repolarized cells after omitting calcium from the external medium and after blocking the cell's calcium channels with cobalt ions, in two separate experimental setup.

6.2 Materials and Methods

6.2.1 Tissue Culture

Cell suspensions of *Nicotiana tabacum* and *Populus deltoides* were obtained as per the method described in Chapter 2 and subcultured on medium containing 0.1 mg/l kinetin and 1.0 mg/l 2,4-D. These subcultures gave rise to cells with weak or random electrical polarities. The transcellular currents of these cells tend to be random and becomes easily displaced in the external electric fields (Mina and Goldsworthy, 1991).

The measurement of transcelluar current in cells were carried out on media with or without calcium and in presence of calcium with calcium inhibitor Cobalt chloride (2.0 mM i.e. 0.5 mg/l).

6.2.2 Current measurements

Transcellular currents were measured with a vibrating probe originally designed by Jaffe and Nuccitelli (1974) with modifications suggested by Goldsworthy and Mina (1991) to give long-term stability. The probes were prepared from stainless steel microelectrodes. The reference electrode was made from about 3.0-cm long stainless steel wire submerged in the medium. The signals from the probe were processed by a lock-in amplifier. The transcellular

currents were measured in small filaments of 5-10 linearly arranged cells sucked in a microcapillary. The mineral component of the suspension medium was a balanced mixture of all the macro and micronutrients needed for plant growth, optimized for the growth of tissue cultures. The mineral content of this medium were reduced by a factor of ten to increase its resistivity (approximately upto 2000 ohm cm^{-1}). The complete composition of the medium is described in the Chapter 2, especially the medium containing 0.1 mM calcium for current measurements.

6.2.3 Current Application

After the initial currents of the filament were measured, an artificial current was passed across the filament axis. The current was applied between a positive Ag/AgCl microelectrode in a capillary (tip diameter 5 μm) filled with 3.0 M KCl, placed 35 μm from the filament on one side, and a negative stainless steel electrode approximately 3.0 cm. away the other end of filament. The current diverged uniformly in the region immediately surrounding the microelectrode and its density in the medium followed the inverse square law (Schnepf, 1986). The current density was 250 μA cm^{-2} at the surface of the cell nearest to the microelectrode and about 35 μA cm^{-2} at surface of the farthest cells. However, the exact value of the current densities depends on the size of the cell. The potential difference across the whole cell was found to be in the range of 3.0 mV. After the

application of current, the microelectrode was removed to prevent its interference with the measurements, and the cell's transcellular currents were measured again.

6.4 Results

6.4.1 The pattern of current

When the pattern of current flow for the whole filament was mapped before and after the application of the external current, significant changes were found only in the cell immediately adjacent to the microelectrode (Fig. 6.1). The current remained highly localized in the region of their entry into the cell, which were nearest the positive electrode. There was no corresponding increase in current density leaving at the diametrically opposite point of the cells. Instead, the extra current after its entry appeared to become uniformly spread over the cell surface.

To quantify the results, the measurements were made on the electrical polarization due to increase in current at entry point in the tobacco and poplar cells nearest to the positive electrode. To confirm that the current increase was not due to a general (i.e. non-polar) stimulation of transcellular currents, similar measurements were also made at the diametrically opposite points on the cells. Changes due to the application of external currents, were negligible and statistically insignificant, thus confirming that the stimulation of current entry nearest the positive electrode had a specific effect on cell polarity rather than a general stimulation of transcellular currents.

6.4.2 Effect of omitting calcium

Table 6.1 shows the effects of the exogenously applied current on cells in the presence and absence of calcium. No special attempt was made to sequester calcium in the minus-calcium treatment. However, the bathing medium contained 0.01 mM EDTA as a normal constituent of the medium, which could make a similar effect. The initial measurements of the transcellular currents were made immediately after the cells were transferred to the calcium negative medium. The transcellular current were found to be of similar magnitude and pattern to those observed for cells maintained in calcium positive medium (Table 6.1). This suggested that the current flow were not greatly affected by the calcium and the movement of this ion was probably not coupled with of the transcellular current.

When an external current of 250 $\mu A\ cm^{-2}$ was applied for 10 minutes in absence of calcium, there was no significant change in the transcellular current at any point on the cell (Table 6.1). It was interesting to find the stimulation of the current-entry, normally found next to is the positive electrode, was absent. Although the currents themselves had not been inhibited, they lost their ability of reorientation in the applied field. This suggests that calcium ions are needed for this reorientation.

The requirement for the electrical repolarization of the cells was confirmed by substituting the bathing medium with one containing 0.1-mM calcium chloride. No significant change was found in either pattern or magnitude of the transcellular currents due to the presence of calcium. But when the external current was re-applied for 10 minutes the cells did show a change in electrical

polarity with a significant stimulation of the current uptake at the entry point located nearest to the positive electrode. This confirms that calcium is needed to control the reorientation of the cell's electrical polarity in an electric field.

6.4.3 The effect of cobalt ions

When 2.0 mM cobalt chloride was added in the medium having 0.1 mM calcium, cobalt had relatively little short-term effect on the magnitude of the cell's *natural* transcellular currents (Table 6.3), suggesting that calcium is not a major component of the current itself. Experiments were then performed to study the effects of cobalt ions on the ability of the cells to repolarize in response to an applied electric current. Filaments were suspended in a culture medium containing 0.1 mM calcium ions and 2.0 cobalt chloride. After approximately 1 hour of equilibration, their initial transcellular currents were measured. A transverse current of 250 $\mu A.cm^{-2}$ was then applied across the filament for 10 minutes and the transcellular currents remeasured. The results are shown in Table 6.2 and 6.3. From the table it is evident that there was no significant increase in current entry in the cells on the side nearest to the positive electrode in presence of cobalt, but the repolarized in the normal way. It therefore seems likely that the electrical repolarization of these cells in electrical fields depends on the proper functioning of calcium channels.

6.5 Discussion

The present investigation reveals that the density of exogenous current remain high at the point of entry into the cell and thereafter becomes diffused throughout the cell even at the point of leaving the cells. In other words, transcellular current flow is always asymmetric when culture cells are put into an exogenous electric field.

Earlier Peng and Jaffe (1976) concluded that the asymmetric current pattern of the electrically treated cells is due to the higher current density on the side of the cell nearest the microelectrode, which damage the membrane to allow greater current ingress. But the potential difference generated is too low (3.0 mV across the whole cell) to cause dielectric breakdown of the membrane. Mina and Goldsworthy (1991) performed experiments, where the microelectrode was made negative. They found that the local increase in current entering occurred on the side furthest from the microelectrode where the current density was lowest. However, the site of current ingress was still on the side of the cell which was nearest the positive electrode, indicating that the polarity of the new transcellular current depended on the polarity of the current applied and not its density gradient.

The present observations on the pattern of the repolarizing tobacco and poplar cell also has remarkable resemblance to the electrical pattern of the polarizing fucoid zygote, where current entry is highly localized and diffuse at the point of entry (Nuccitelli, 1978).

The finding that calcium is important in controlling cell polarity is in agreement with the observations of many other workers (Quatrano, 1978 and Schnepf, 1986). Since calcium ions are rapidly pumped out of the cells and adsorb to negatively charged macromolecules, the cells exterior contain very little free calcium, mostly concentrated near the site of entry. Such localized calcium accumulations seem to control polar growth as revealed by the autoradiography on the growing tip of pollen tubes (Jaffe et al., 1975). It has also been reported that cell polarity can be induced in a calcium gradient imposed artificially by the ionophore in *Pelvetia* zygotes (Chen and Jaffe, 1979; Robinson and Cone, 1980) The polar growth of the fucoid rhizoid is associated with a natural intracellular gradient of free calcium (Brownlee, 1986).

The mechanism by which calcium induces growth is complex. It is well established that it regulates plant development by acting as a second messenger to interact with a variety of enzyme systems as well as by controling transmembrane movement of other ions via calcium-gated ion channels (Helper and Wayne, 1985; Marme, 1988). The present work suggests that external calcium forms part of the mechanism by which higher plant cells perceive external electric fields to reorientate their own electrical polarities.

The importance of calcium in the initial establishment of electrical polarity in the fucoid zygote is already evident from the observation by Robinson and Jaffe (1975). According to these worker, the calcium ions form a substantial but declining proportion of the early transcellular current entering the presumptive growing region. This may be interpreted as an initial localized entry of calcium

stimulating the activity of channels for other ions. If so, it seemed possible that the necessary calcium might be entering via specific calcium channels. If this was the case, calcium channel blocked by cobalt salts (Weakly, 1973) should inhibit the electrical repolarization of the cells. The present observations on the effect of cobalt chloride in *Nicotiana tabacum* and *Populus deltoides* are in conformity with the above observation of Weakly (1973).

Table 6.1 Effect of an external current of 250 μA. cm^{-2} applied for 10 min. to *Nicotiana tabacum* and *Populus deltoides* cells (average of 25 replicates)

	Transcellular currents (μA.cm^{-2})			
	Minus calcium		Plus calcium	
	Initial	After current	Initial	After current
N. tabacum	+0.017 ±0.005	+0.016 ±0.002	+0.008 ±0.002	+0.066 ±0.016
P. deltoides	+0.026 ±0.009	+0.023 ±0.012	+0.019 ±0.002	+0.093 ±0.017

Table 6.2 Effect of an external current of 250 μA. cm^{-2} applied for 10 min. to *Nicotiana tabacum* and *Populus deltoides* cells before and after the addition of $CoCl_2$ (2.0 mM)
(average of 25 replicates)

	Transcellular currents (μA.cm^{-2})		
	Before addition of $CoCl_2$	Time after addition of $CoCl_2$ 2hours	15 hours
N. tabacum	0.085 ±0.017	0.073 ±0.015	0.021 ±0.003
P. deltoides	0.127 ±0.20	0.096 ±0.018	0.034 ±0.008

Table 6.3 Transcellular currents of *Nicotiana tabacum* and *Populus deltoides* cells before and after the application of an electric current of 250 μA. cm^{-2} for 10 min. in presence of calcium and presence and absence of $CoCl_2$ (2.0 mM) (average of 25 replicates)

Treatments	Transcellular currents (μA.cm^{-2}) Initial	After current application
Nicotiana tabacum		
Minus cobalt	-0.085±0.014	+0.126±0.048[1]
Plus cobalt	-0.089±0.027	-0.067±0.051
Populus deltoides		
Minus cobalt	-0.118±0.036	-0.203±0.073[1]
Plus cobalt	-0.115±0.085	-0.108±0.057

[1] Significant at $p<0.01$

Chapter 7

Conclusions

In the present investigation, experiments were carried out to examine the effects of externally applied weak electric current on the augmentation of growth and differentiation in undifferentiated, differentiated and embryogenic calli of *Nicotiana tabacum* and *Populus deltoides*. The external current was applied to the above tissues cultured in media with or without calcium ions and calcium inhibitor.

The measurement of natural surface electrical potential in undifferentiated and organogenic calli prior to the application of external electric current reveals prevalence of specific natural electric potential pattern during their growth and development. The present observations concludes that (1) a minimum electrical potential required for differentiation in both *Nicotiana tabacum* and *Populus deltoides* is 60 mV negative to the medium, (2) above this potential, the tissues undergo regeneration and organogenesis, (3) below 60 mV the differentiation process decline progressively and only undifferentiated growth occurs below 20 mV potential, and (4) there is no relationship between natural potential of callus tissue and embryogenesis.

The above observation also suggests that if exogenous electrical current is passed through the callus having low natural potential regions, the undifferentiated tissue can be brought to the differentiation regime. This should induce the differentiation process in otherwise undifferentiated tissue.

An important finding of this investigation is the high sensitivity of cells to external electric fields in the unpolarized callus growth. The applied potential across the entire cell was only 3mV. This is of the same order as the voltages needed to polarize the growth of the fucoid zygote (Peng and Jaffe, 1976) and *Funaria* spores (Chen and Jaffe, 1979). Even higher sensitivities have been reported for tobacco cells (Mina and Goldsworthy, 1991) with electrical polarization occurring in approximately 100 times lower fields when applied for several hours.

Because of their high resistance, most of the applied voltage appears across the cell membranes where it adds to or subtracts from the normal membrane potential. The membrane on the side of the cell nearest the positive electrode becomes *hyperpolarized*, whereas that on the opposite side becomes *hypopolarized*. Since the applied voltage amounts at most to a few per cent of a typical membrane potential and falls well within its natural range of variation, it is unlikely that the local stimulation of current entry by the external electric field is due to the opening of the voltage-gated ion channels at a specific threshold (Scott and Martin, 1962; Chen and Jaffe, 1979; Deloof, 1986; Mina and Goldsworthy, 1991).

The results of experiments reveals that the application of 1μ A and 2 μ A external current of both polarity, influenced both the undifferentiated and differentiated growth. The negative polarity of 1μA and 2 μA currents augments the callus growth and organogenesis. These results leads to the conclusion that application of external electric current reorients the natural potential, and

influence the uptake of auxin, i.e. IAA, which is well known growth regulator for induction of cell division and cell elongation.

The cytomorphological analysis of the effects of exogenous electric current on the growing calli, demonstrates that the number of cells per gram of fresh tissue doubled on the negative polarity treatment. Further the cells become more and more enlarged rather than remaining circular with smaller volume. Thus it may safely be concluded that the application of external electric current augments differentiation and undifferentiated growth in calli by enhancing the rate of cell division and cellular enlargement.

The behavior of transcellular current flow during the passing of exogenous current was observed in tissue cultured on media with and without calcium ions, and with calcium inhibitors. From the pattern of current observed it might be concluded that the reorientation of natural electric potentials under influence of external electric current is governed by the presence of calcium.

The cell appear to have a very sensitive mechanism which continuously compares the membrane potential simultaneously in all regions and uses relatively minor differences to stimulate massive current entry in a relatively small area where the membrane is most hyperpolarized. A simple hypothesis to explain the findings is that the cell membrane contains channels which allow the entry of calcium ions, with more calcium entering along the steeper voltage gradient on the hyperpolarized side of the cell (i.e. nearest the positive electrode). This effect has already been demonstrated in fucoid zygote where calcium influx is more or less proportional to the membrane potential (Chen and Jaffe, 1978; Nuccitelli,

1988; Deloof, 1986; Harold, 1986). The differential current entry carried by calcium on either side of electrically treated cells must, however, be minute. Nevertheless, it could be amplified if it opened calcium-gated channels for other ions, perhaps as in the fucoid zygote where chloride efflux is stimulated by calcium entry (Nuccitelli and Jaffe, 1976). The effect of this would be to promote larger entries of current carried by other ions, roughly in proportion to the local calcium concentration beneath the membrane. These currents would still not have the sharp highly localized and semi-permanent pattern was observed in tobacco by Mina and Goldsworthy (1991), and also in the fucoid zygote (Lund, 1947). However, it would enable this pattern to be achieved if there were to be a second stage in amplification involving the movement, perhaps by the lateral electrophoresis, of electrically-charged ion channels as proposed by Nuccitelli (1978). The current, after its initial amplification, would generate a relatively smooth potential gradient along the membrane which reflects the original calcium concentration and be strong enough to allow charged ion channels to migrate along it. They could move up the gradient to its peak, where they would accumulate and become fixed to the cytoskeleton. This would cause a large relatively sharp and quasi-permanent increase in membrane conductance and current-inflow at this point. The experiments suggest that the net effect would be to concentrate current-entry in a relatively small region next to the positive electrode, leaving current-efflux relatively undisturbed and widely dispersed.

This hypothesis also predicts that any ion channels migrating electrophoretically in this way must use mainly the voltage gradient on the inner

surface of the membrane as the driving force. If they used the gradient on the outer surface, they would have moved directly in the applied field and the cell repolarization would not depend on calcium and functional calcium channels. This is also to be expected on theoretical grounds, since the charge on a protein is highly dependant on pH and only the internal surface of the membrane is tightly controlled. Consequently, protein-based channels could only move reliably by electrophoresis using the charge at their inner ends. When these channels are isolated, it would be expected that their external surface will be uncharged, but their internal surface to carry a strong negative charge at physiological pH so that they would be attracted to the site of entry of positive ions.

Whatever the precise mechanism, on the basis of evidence presented in the present investigation, it can be inferred that the repolarization of higher plant cells in response to external electric currents seems to be similar in current pattern and ionic mechanism to the initial polarization of the fucoid zygote (Lund, 1947). These levels are more commensurate with those of the currents found naturally in the living tissues and this extra sensitivity could enable these cells (which normally grow as a part of a tissue) to entrain and co-ordinate their polarity with those of their neighbors to form organized structures. Despite their differing sensitivities, the similarities between the electrical behavior of tobacco and poplar cells, and the fucoid zygote suggest that they may become polarized by the fundamental basis upon which the majority of plants organize and co-ordinate the polarity of their component cells so that they align properly to form tissues and organs.

The above discussion on the of results obtained in the present investigation lead to the conclusion that the cells of unpolarized callus growth in *Nicotiana tabacum* and *Populus deltoides* are highly sensitive to the external electric fields even lower than 3 mV. The external electric field of 1 μA and 2 μA reorients the natural electric potentials of cells in culture, open the calcium channels and influence auxin uptake. These events inturn enhance the rate of cellular division and enlargement, and induces differentiation and organogenesis.

Summary

The present research work entitled "The Biophysical Mechanisms of Electrical Stimulation on *in vitro* Growth and differentiation in *Populus deltoides* and *Nicotiana tabacum*" has been carried out to investigate the effect of externally applied electric current on growth of unpolarized tissue and differentiation in calli of *Populus deltoides* and *Nicotiana tabacum*, and also the mechanism of action involved. The work has been presented through seven chapters followed by summary and Bibliography/ references.

Chapter 1: Introduction

The chapter includes the survey of literature, aims and objectives of the present investigation.

1. A survey of the earlier work related to the present investigation has been made. The review includes the areas of electrophysiology of plants and tissue culture.
2. Aims and Objectives of the present investigation have been defined along with the parameters investigated to achieve the aim.

Chapter 2: Plant Tissue Culture Protocols

Experimental plant materials for the present investigations were pure line seeds of *Nicotiana tabacum* and axillary buds of *Populus deltoides*. The seeds of *N. tabacum* and axillary bud explants were surface sterilized before inoculating

them in cultures. Culture media used in the investigation were Gamborg's B5 medium, Linsmair and Skoog medium and White's media. They were prepared and autoclaved before inoculating the explants. Culture establishment and maintenance methodologies were described. The surface sterilized explants of both the plants were inoculated and incubated for growth. The cultures were incubated under 3 k.lux cool florescent light for 14-16 hrs. at 70% RH and 27±1 °C for 20-45 days. Growth Regulators supplement in various combinations and concentrations in media were described. The calli were initiated from excised hypocotyl of *N. tabacum* seedlings and leaves segments of *P. deltoides*. The undifferentiated growth was induced by 2.0 mg/l 2,4-D and 0.3 mg/l kinetin in *N. tabacum*, and 2.0 mg/l 2,4-D and 0.25 mg/l BAP in *P. deltoides*. The shoot proliferation in *N. tabacum* medium was supplemented with growth regulators 15 mg/l AdS and 1.0 mg/l IAA, and in *P. deltoides* 15 mg/l AdS, 1.0 mg/l IAA and 0.25 BAP. The differentiation in undifferentiated tissue in both the plants, 1.0 mg/l kinetin and 0.3 mg/l IAA combination was used. The generation of embryo the BM was supplemented with 0.1 mg/l 2,4-D and 0.1 mg/l kinetin for both the plants. Cell suspension culture for both plants were generated in media with 0.1 mg/l kinetin and 1.0 mg/l 2,4-D. The transcellular current were measured for various tissue in their respective medium.

Chapter 3: Electrical Control of differentiation in Callus Natural Potential

The callus were cultured and maintained at 25 °C under 3.0 klux fluorescent illumination for 5-6 weeks. Silver electrodes were prepared by putting 0.125 cm diameter and 40 mm long silver wires in 1.0 mM KCl solution and thereafter sealing them a flexible plastic pipettes tip filled with 3.0 mM KCl. Electrical Measurements were made by inserting one electrode in culture media and other in the center of the growing culture and using Bachman HD153 digital multimeter. The surface potential measured for the callus tissue for both the plantsat various sites on calli surface were typically <10 mV and never >15 mV during undifferentiated growth. When the callus were grown on shoot forming media under illumination the surface potential were beyond 60-100 mV negative to the culture medium and give rise to green dense area on callus. Embryogenesis in *P. deltoides* did not indicate any dependence with natural potential

Chapter 4: Electrical control of growth in Plant tissue culture

This chapter records and concludes the effect the of application of external 1μA and 2 μA electrical current to the calli of both the plants. The electrical current of said current levels were passed to the callus through the stainless steal electrodes,

resistors and d.c. supply. The result in each polarity was tested. The callus negative treatments were found to be more conducive than the callus positive treatments, which was very much similar to the controls. The growth pattern in absence of growth factor IAA was analyzed and found that in absence of IAA or in presence of TIBA (inhibitor of IAA uptake) the electrical current do not bring the enhancement in growth. The results indicate the enhanced growth under electrical influence was because of polar uptake of IAA. The regeneration process is also augmented by the electrical influence. The results were significant at level of $p<0.01,0.001$ and 0.05.

Chapter 5: Electrical Control of Growth: Cytomorphological evidence to the phenomenon

Since the 1 μA current level is more conducive to growth as compared to 2 μA the result were discussed for the 1 μA current level. The cell number per gram fresh weight or packed cell volume (PCV), cell shape index and cell volume were determined for each treatments. Cell volumes were calculated for circular cells and elongated cells by using solid geometry techniques. It was found that the PCV is increased to $8.29X10^6$ cells from $4.4X10^6$ cells by the application of 1 μA current. Cell elongation rate is also enhanced under the influence of external electric current. These results indicate that exogenous current alters the unpolarized tissue growth into polarized growth by regulating the unidirectional auxin flow.

Chapter 6:Electrical Polarization of Cells by calcium ion channels

The transcellular currents were measured in cell growing in suspension media with and without calcium, and with both calcium and its inhibitor cobalt chloride. The current densities on the cell surface near the microelectrodes were 250 μA cm^{-2} but was 35 μA cm^{-2} at the cell surface of the farthest from microelectrodes. By increasing and decreasing the external current density at the point of entry, it was found that there is an electrical polarization of the cells. Measurement of electric potential in absence of calcium in the culture medium, were similar to those recorded in presence of calcium ions but the cell looses their ability of reorientation. The later was restored on adding calcium ions into the medium. The addition of cobalt chloride in culture media with calcium ions also resulted in the loss of cellular reorientation ability. The results indicates that external current open the calcium gated channels in polarized state for transport of biomolecules.

Chapter 7: Conclusion

The results obtained from the various experiment were discussed and correlated. The measurement of natural electric current suggests that minimum electrical potential required for the differentiation of tissues is 60 mV negative to the medium. Cells in unpolarized callus growth are highly sensitive to external electric current. The external electric field reorients the cellular polarity for unidirectional uptake and flow of auxin which enhance the growth. The

cytomorphological analysis concludes that the application of external electric current enhances differentiation and growth in calli by enhancing the rate of cell division and cellular enlargement. The reorientation of natural electric potential of cell surfaces by external current is governed by presence of calcium in both *N. tabacum* and *Populus deltoides*.

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