Induction of Programmed cell death in tobacco Bright Yellow-2 cell lines and *Saccharomyces cerevisiae* strain*YPH-500* against various apoptotic stimuli

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CERTIFICATE

This is to certify that the thesis entitled "**Induction of Programmed cell death in tobacco Bright Yellow-2 cell lines and *Saccharomyces cerevisiae* strain*YPH-500* against various apoptotic stimuli"** entitled submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy (Plant Biotechnology)** of the University of Agricultural Sciences, GKVK, Bangalore, is a bonafide record of research work done by **Mr. Lakshmana Reddy. D.C., ID No. PAK 3054,** during the period his study in this University under my guidance and supervision and thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Place: Bangalore
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THESIS ABSTRACT

Induction of Programmed cell death in Tobacco Bright Yellow-2 cell lines and *Saccharomyces cerevisiae* strain YPH-500 against various apoptotic stimuli.

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Programmed cell death (PCD) is a fundamental cellular process conserved in metazoans, plants and yeast. Our results suggested that hydrogen peroxide, heat and salinity induces PCD in tobacco bright yellow-2 cell lines and yeast strain YPH 500; these results are comparable to earlier reports. In tobacco BY-2 cell lines, hydrogen peroxide induces programmed cell death at 10mM concentration and typical features of PCD like membrane blebbing, DNA fragmentation, ROS rise and MMP loss were observed. Vacuole morphology varied during PCD process against H_2O_2, heat and salinity stresses.

PCD observed in yeast against H_2O_2, heat and salinity stresses with typical hallmarks like DNA fragmentation, ROS rise and MMP loss. The concentration of H_2O_2 required in yeast is lower than the tobacco cells. In case of salinity, yeast requires higher (1.5M) as against 200mM in tobacco cells; there is no difference in induction of PCD with respect to heat stress in both systems. The death percentages were reduced by pre incubating yeast cells with calcium and ROS chelator and scavengers respectively.

In yeast cells, elevation of ROS levels was observed against NaCl and heat stress and the elevation of cytosolic calcium levels was observed against H_2O_2 and NaCl stress. The rise of calcium levels was reduced by pre incubation with EGTA. The rise of calcium levels is less in H_2O_2 stress compare to saline stress. The reduction in percentage of cell death was observed in yeast cells against H_2O_2, Heat and NaCl by preincubation with scavengers. TEMPOL and Glutathione reduce cell death against H_2O_2 and Heat, where as EGTA reduces cell death against H_2O_2, Heat and NaCl.

In our study, results conclude, both tobacco BY-2 cell and yeast cells exhibit substantially similar PCD characters against H_2O_2, Heat and NaCl stresses.

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ABBREVIATIONS

°C	degree Celsius
ABA	Abscisic Acid
AMP	Adenosine-5'-monophosphate
AO	Alternative Oxidase
ATP	Adenosine-5'-triphosphate
BH_4	Tetrahydrobiopterin
CaMV 35S	Cauliflower Mosaic Virus 35S promoter
cDNA	Complementary DNA
cGMP	cyclic Guanosine 3´,5´-Monophosphate
CHX	Cycloheximide
cNR	constitutive Nitrate Reductase
c-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl
DAF-2	4,5-diaminofluorescein
DAF-2DA	4,5-diaminofluorescein diacetate
DAF-2T	4,5-diaminofluorescein triazole
dATP	deoxyadenosine-5'-triphosphate
DEPC	dietyl pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	2'-desoxyribonucleosid-5'-triphosphate
EDRF	Endothelium-Derived Relaxing Factor
EDTA	Ethylenediamine-tetraacetate
e.g.	*exempli gratia* (= for example)
eNOS	endothelial Nitric Oxide Synthase
FAD	Flavin Adenine Nucleotide
FMN	Flavin Mononucleotide
GC	Guanylate Cyclase
GDC	Glycine Decarboxylase
h	hour

HEPES	N-2-hydrovyethylpiperazine-N'-2-ethanesulfonic acid
HR	Hypersensitive Response
iNOS	inducible Nitric Oxide Synthase
iNR	inducible Nitrate Reductase
JA	Jasmonic Acid
kb	kilo base
L	Liter
Lb	Leghemoglobin
L-NAME	N ω -nitro- L - arginine methyl ester hydrochloride
L-NIL	L-N^6-(1-Iminoethyl)-lysine, acetate
L-NMMA	N^G-Monomethyl-L-arginine, Monoacetate salt
M	Molar
MAPK	Mitogen Activated Protein Kinase
max	maximal
MES	4-morpholinic-ethanesulfonic acid
mg	milligram
min	minutes
mL	milliliter
mm	millimeter
mM	millimolar
MOPS	4-morpholinic-propansulfonic acid
mRNA	messenger RNA
NAD^+	Nicotinamide adenine dinucleotide (oxydized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NR	Nitrate Reductase

NRA	Nitrate Reductase Activity
PAL	Phenylalanine Ammonia Lyase
PCD	Programmed Cell Death
pH	Potential of Hydrogen
PM	Plasma Membrane
pmol	picomol = 10^{-12} mol
PR	Pathogenesis related
PTIO	2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl
PVP	Polyvinyl pirrolidone
ROS	Reactive Oxygen Species
rpm	rounds per minute
RSNO	S-nitrosothiols
RT	Room Temperature
s	second
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
XOR	Xanthine Oxidoreductase

Introduction

I. INTRODUCTION

Apoptosis or PCD is an active form of cell death and occurs in multicellular /pluricellular organisms for maintenance, homeostasis and defense. This process is highly complicated and needs a co-ordinated action of a large number of gene products. In mammals /plants, PCD can be induced either by external or internal stimuli and occurs via several signaling pathways. These stimulants are spread over a wide range, right from viral toxins to protein synthesis inhibitors, biotic and abiotic stresses.

This process is differentiated by phenotypic, morphological and molecular characters like exposure of phosphotidyl serine on the cell surface, condensation of chromatin and the nuclear envelope. DNA fragmentation and formation of membrane enclosed cell fragments called apoptotic bodies in animal systems (Kerr *et.al, 1972).* The entire process divided into various phases via Induction, Transduction and Execution phases in animal systems.

Programmed cell death is an integral part of plant development, homeostasis and defense and it is known occur at all stages of the life cycle of plants from fertilization to senescence. The plant PCD is categorized into three types *viz* Apoptosis, autophagy and non–lysosomal PCD in relation to mammalian morphological types of cell death (Donon *et al*., 2005). But most examples of plant PCD conform either to the autophagic or apoptotic type.

Saccharomyces cerevisiae (budding yeast) has been successfully used as a model to understand physiological process of metazoan cells and some fundamental results have been achieved in the fields of aging, vesicular transport, cell division etc. For a long time,there has been debate as to whether there is presence or absence of apoptosis in unicellular organisms and it was not well documented. However, the occurrence of an apoptosis like process as reported in

phenotype associated with a specific mutation in *S.cerevisicae* (Madeo *et al., 1999)*.CDC48 mutant showed typical hall marks of apoptosis like exposure of phosphatidyl serine (PS), chromatin condensation and fragmentation and these basic hall marks of apoptosis were also detected in yeast cell death induced by acetic acid, viral proteins, hydrogen peroxide, etc.

The discovery of an apoptotic phenotype in a yeast strain carrying cdc48 mutant was unexpected and leads to believe the occurance of apoptosis in yeast. This later aided to discover several mammalian gene orthologues and homologues of apoptotic regulators like Yca1, AIF, Omtr, EndoG etc in yeast system and also helps to understand basic process of ROS mediated signaling pathway in apoptotic process.

Calcium is a well known second messenger in animals, plant and yeast systems (Sanders *et al*., 2002). The Ca^{2+} functions as a ubiquitous intracellular messenger in animals and the extracellular factors induce cytosolic calcium rise resulting in a variety of physiological responses such as proliferation, muscle contraction etc. in animal systems.

The calcium rise in cytosol results in an array of signals and responses in plants like stomatal behaviour, root gravitropism, salt tolerance and osmotic signaling in yeast. These responses and signals are mediated by a proteins such as calmodulin,Ca^{2+} dependent protein kinases and calcium binding proteins (Reddy *et al*., 2002,Zheng *et al*., 2007)

The rise of calcium at cytosol occurs either by influx from calcium stores like vacuoles, ER, mitochondria and plasma membrane or by decreasing calcium efflux. This process is mediated by various calcium permeable channels (Sanders *et al*., 2002) like PM depolarization activated Ca^{2+} channels (DACCs), which

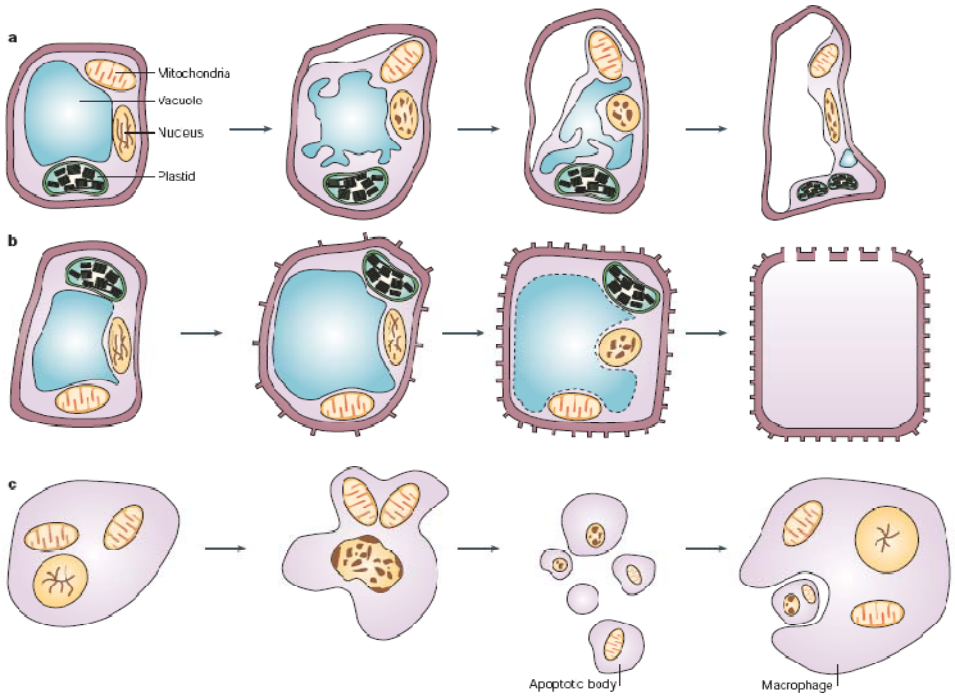


Fig. 1. Morphological comparisons between Plant PCD and animal Apoptosis.

a. In the hypersensitive response, chromatin condensation and DNA cleavage into 50-kb fragments.

b. During the differentiation of treachery elements, vacuole swelling and rupture is coordinated with the thickening and restructuring of the cell wall.

c. Apoptosis in animal cells initiates morphologically with chromatin condensation and fragmentation.

Ref. LAM, E., 2004

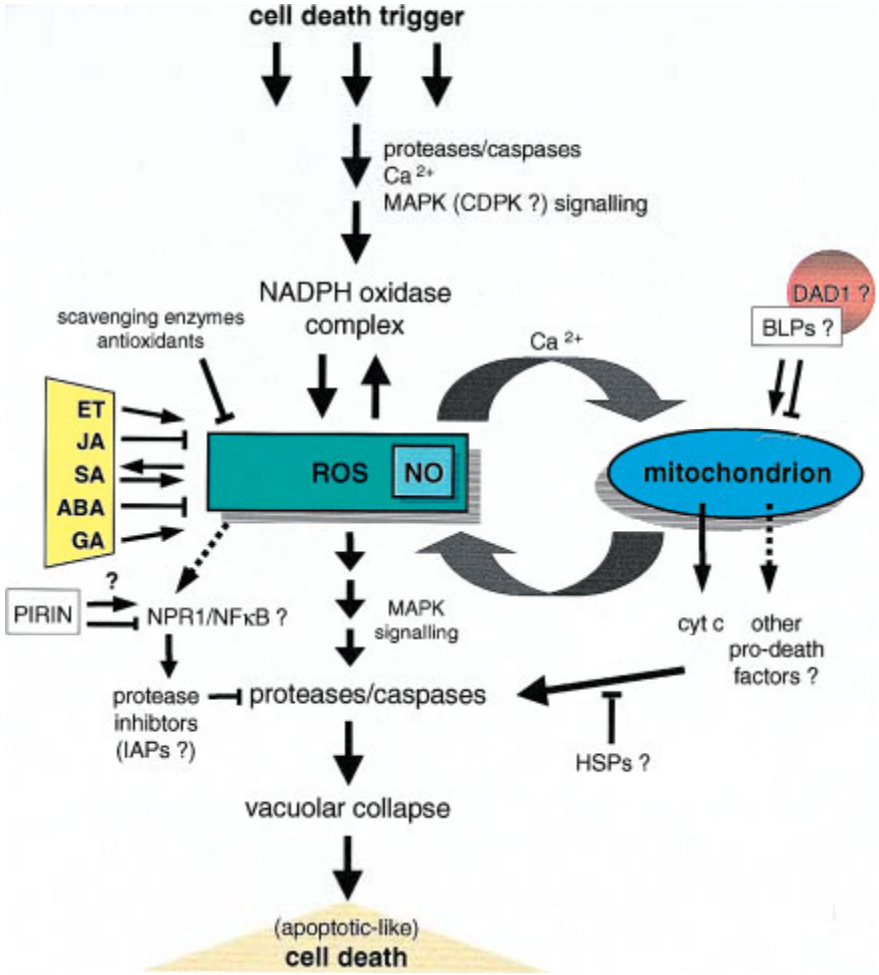


Fig. 2. Model of Plant programmed cell death

Ref: Hoeberichts,F.A. and Woltering, E.J., 2003

involves in rise of cytosolic calcium during biotic and abiotic stimuli in plant systems.

The change in cytosolic concentration of free calcium plays a role in regulating apoptosis in animal cells and it was implicated as an early event in apoptosis. Kroemer (1995) reported that increase in $(Ca^{2+})_{cyt}$ levels causes opening of MPTP and loss of $\Delta\psi_{m,}$ which results in leakage of apoptogenic factors from the inner mitochondrial membrane space into the cytoplasm in animal system. It is also reported that elevation of Ca^{2+} results in activation of wide variety of Ca^{2+} sensitive enzymes, which generates signalling molecules for recruitment of mitochondria to the apoptotic cascade or activation of the caspase enzymes in animal systems.

Knight et al., (1996) reported that changes in calcium $(Ca^{2+)}_{cyt}$ levels increases adaptive responses to various stress conditions like light, hormones, gravity, touch, wind, cold, drought, oxidative stress and fungal elicitors in plant cells. Recently, several lines of evidence suggest that changes in $(Ca^{2+})_{cyt}$ might be associated with PCD. Okadaic acid (Protein Phosphatase Inhibitor) blocks both Ca^{2+} changes and Gibberlin induced PCD in aleurone cells. Ca^{2+} rise is also reported in HR-Elicitation response during defense gene activation (Levine *et al.,* 1996).

In resting yeast cells, the $(Ca^{2+})_{cyt}$ concentration is in the range of 50-200nM. When cells are exposed to environmental abiotic and biotic stimuli, it will rise upto 1μM to 100mM. The rise of cytosolic Ca^{2+} observed under the pheromone stimulation(Muller *et al*., 2001), hypertonic shock (Batziaa *et al*., 1996), NaCl stress (Matsumato *et al*., 2002). But the Ca^{2+} homeostasis in a yeast cell involves the variety of intracellular compartments such as ER, Golgi apparatus through many membrane proteins.

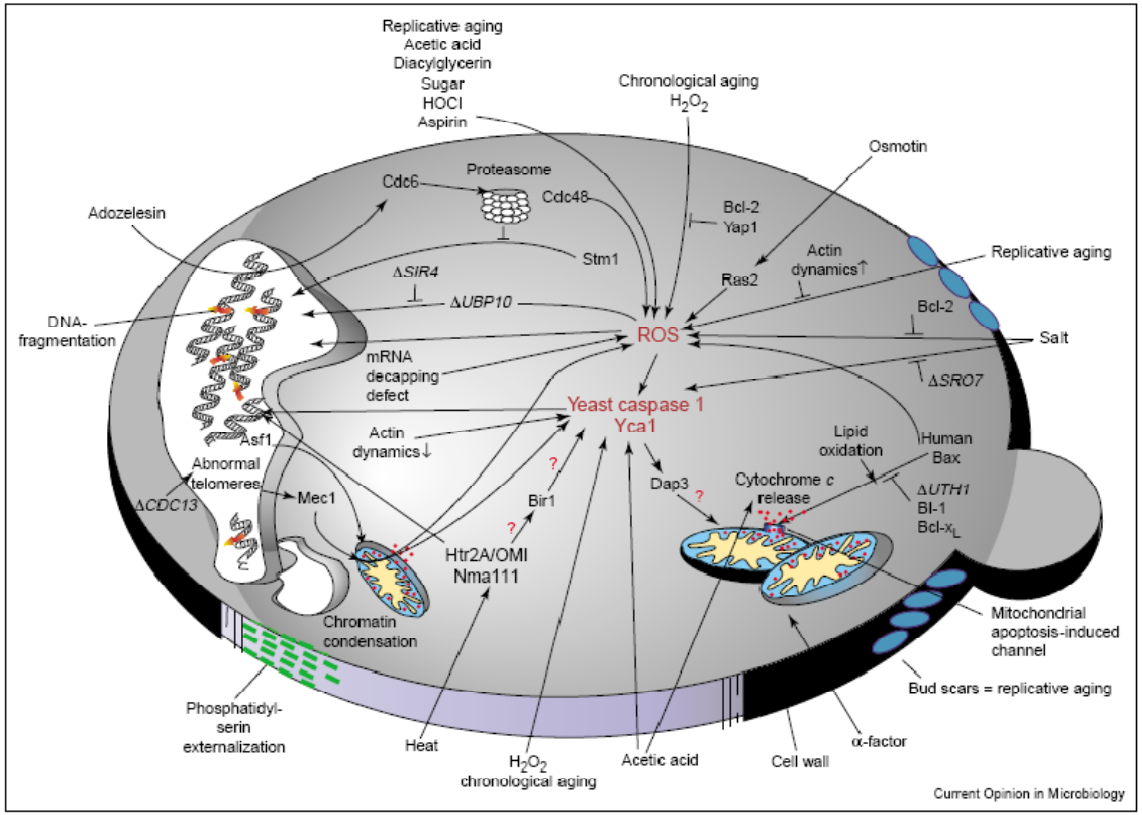


Fig. 3. Apoptosis in yeast, the key players in apoptosis and their interdependence
Ref; Madeo *et al*., 2002

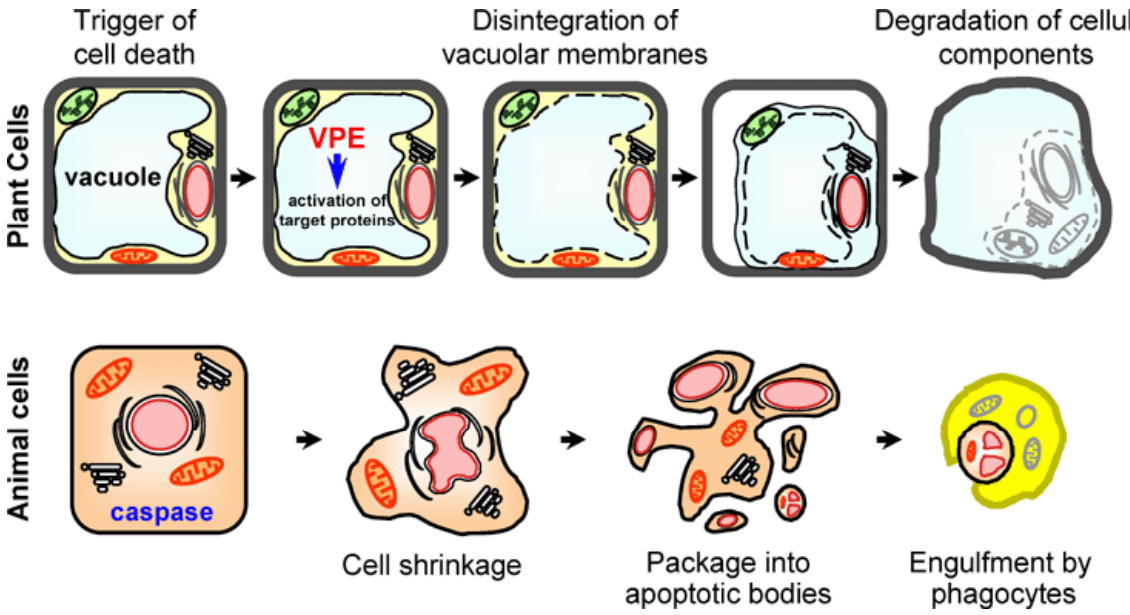


Fig. 4. VPE processing system mediates a cellular suicide strategy in plants.
Ref:Lam E, *Trends cell Biol*,2005

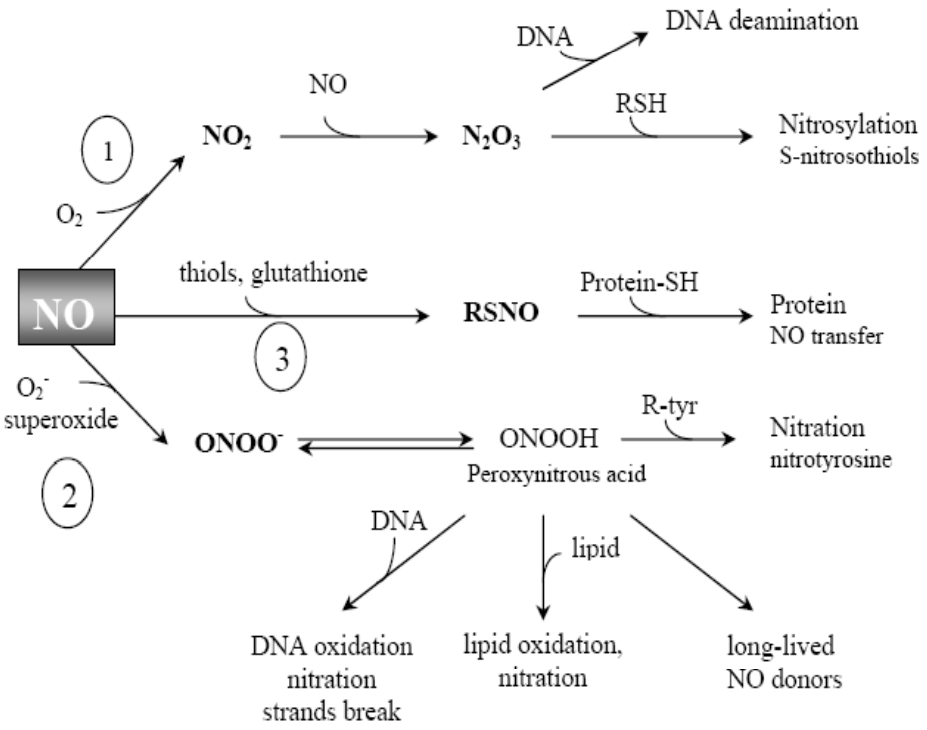


Fig. 5. Reactions of the free radical NO and reactive nitrogen oxide species.
Ref. Grubisic and Konjevic, 1992

Several studies revealed that reactive oxygen species (ROS) or reactive oxygen intermediates (ROI) concentrations are altered after various death stimuli in animals, plants and yeast. The changes in concentrations of ROS are perceived and transferred into signals to change physiological processes like membrane damage, the regulation of transcription factors and ROS mediated signaling mechanisms. Among these ROS, hydrogen peroxide (H_2O_2) and superoxide signaling pathways are implicated to have prominent roles in abiotic and biotic stress responses. The responses include defense reactions against pathogens, herbivores, stomata closure and plant development. The elevated ROS concentrations also damage mitochondrial membrane, vacuole tonoplast and other sub cellular organelles (Lamb and Dixion *et al*., 1997).

Madeo *et al.* (1999) proposed ROS are not a byproduct, but a promoter in yeast apoptosis and they showed low doses of H_2O_2 induces apoptosis and higher doses induces necrosis. Super oxide mediates the ageing programme and which is responsible for longevity of yeast cells.

In animal systems, ROS reported has been to be an important participant in apoptotic signal transduction and this signaling pathway is critical to potentiate the Ca^{2+} rise in cytosol. The ROS dependent potentiation of Ca^{2+} signaling has been reported in apoptosis in neuronal differentiation.

This link between ROS and cytosolic calcium rise in plants and yeast PCD is not reported earlier. Therefore we designed the following experiments to answer the ROS and calcium link during plant and yeast PCD process by using Ca^{2+} and ROS scavengers.

1. Induction of PCD in tobacco cells (Bright Yellow-2) and yeast (YPH500) by using H_2O_2, Heat, NaCl and Mannitol.

2. Analysis of apoptotic characters, during induction of apoptotsis by stimuli (H_2O_2, Heat, NaCl and Mannitol), like DNA condensation and fragmentation, ROS rise and mitochondrial permeability loss.
3. Transformation of yeast with apoaequorin gene for analysis of calcium fluctuations during apoptosis.
4. To study the effect of EGTA (calcium scavenger) and TEMPOL (ROS scavenger) on yeast apoptosis.
5. Transformation of tobacco cells with *At*-Vam5 GFP, Bax-inhibitor-GFP, Fimbrin-GFP, Peroxi-EYFP by using gene gun.

II. REVIEW OF LITERATURE

Programmed cell death (PCD) defined as a genetically regulated process of cell suicide, accomplishes a central role in development, homeostasis and integrity of multicellular organisms. The origin, evolution and nature of PCD seem to be as old as the very first cell, because mechanisms controlling homeostasis and preventing self-destruction of cells are involved in the PCD machinery (Ameisen, 2002) and this process indeed has been recognized in several prokaryotes and unicellular eukaryotes (yeast). Now this process is referred to as a fine tuned programme in higher organisms compare to unicellular systems. The following aspects with respect to plant PCD and yeast PCD are reviewed in this chapter.

2.1 Morphological and Biochemical Characteristics of Programmed Cell Death.

2.1.1 Plasma Membrane (PM) characteristics –Blebbing and externalization of phosphatidyal Serine (PS).

Apoptosis is a complex phenomenon, which is divided into three phases *viz* initiation phase, transduction phase and execution phase. The distinct morphological transformation is one of the earliest described and most obvious aspects of apoptosis in a cell. This is characterized by events that occur during apoptotic death including cell contraction, dynamic membrane blebbing and DNA fragmentation. These events are well characterized in animal, plant and yeast cells.

In resting cells, the phosphatidylserine(PS) is sequestered in the inner leaflet of the plasma membrane. In a response to a death stimulus, plasma membrane asymmetry is lost and phosphatidyl serine is translocated to the exoplasmic leaflet of plasma membrane. This promotes the assembly and activation of several key enzyme complexes involved in recognition of apoptotic and senescent cells by phagocytes (Fadok *et al.*, 1992). PS externalization was

also reported in tobacco protoplast cells (O'Brien *et al.*, 2006) and in yeast (Madeo *et al.*, 1997) against various death stimuli.

2.1.2 Nuclear condensation and fragmentation

The nuclear morphological features of an apoptotic cell were detected by using cytochemical and microscopic methods. The most extensively used cytochemical methods are staining using propidium iodide (PI), Hoechst 3325 and DAPI of the apoptotic cells, to study nuclear morphology, which mainly helps to detect condensation and marginalization of chromatin in the nucleus (Darzykiwwiks *et al*., 1992).

Plant cells undergoing PCD show characteristic features of fragmentation of DNA occurring at the nucleosomal linker sites and the resulting fragments size varies from 140-200bp. This character is well reported in the dying cells of aleurone layer of barley (Pennel and Lamb, 1997), HR lesions in *Arabidopsis* and tobacco root cap cells (Mitler and Lam, 1997), in wound and herbicide induced PCD (Cutler and sammevillie, 2005).The occurrence of DNA fragmentation is also reported in plants during pathogen attack and it enhances in presence of calcium (Wang *et al*., 1996). But this type of DNA fragmentation is not observed during tracheary elements and fiber differentiation (Mitler and Lam, 1995) or during leaf shape formation (Gunawadena *et al*., 2004).

Like in mammalian and plant apoptosis, the nuclear morphological features of apoptotic nuclei are examined for its condensation and fragmentation by using DAPI fluorescent dye and TUNNEL assays. Heterologus expression of various mammalian pro-apoptotic genes like Bax, bak etc. has showed that yeast exhibits apoptotic nuclear morphological features like condensation and fragmentation (Ink *et al*., 1997, Xu and Reed., 1998).

Initially, Madeo *et al.* (1999) reported that *cdc48* mutant undergoes apoptotic type of cell death with clear nuclear condensation and fragmentation. Similarly to other stimuli, DNA fragmentation was reported by challenge with acetic acid (Ludovico *et al*., 2001), NaCl (Huh *et al*., 2002), sugar (Granot *et al*., 2003) and hyperosmotic conditions (Silva *et al*., 2005).

The other important apoptotic event is the cleavage or fragmentation of DNA into 140-180 base pairs in animals, aleuronic layer cells (Pennel and Lamb., 1997) and yeast (Madeo *et al.*, 1996) during exposure to apoptotic stimuli. Changes in PS were analyzed by using Annexin –V binding assay as detected by flow cytometer or fluorescence microscope.

Morphological changes like cytoplasm shrinkage, condensation and fragmentation were observed, but plasma membrane was always intact. Condensation and shrinkage was reported in aleuronic layer cells (*Wang et al.*, 1996), of *Arabdopsis*, onion and tomato and tobacco cells.

2.2 Caspase like proteolytic enzymes in plant cell death

The key effects of mammalian PCD are cysteine-aspartate specific protease (Caspases), which are critical in the execution phase of apoptosis in mammalian PCD and in *C.elegans* (Thornberry *et al*., 1998). These caspases are activated by adaptor proteins that bind to shared motifs like caspase 8 activated by Death effector domain (DED) (Aron *et al*., 2003). Caspase 9 activation occurs by binding with apoptosis protease activating factor *(Apaf1)* often combined with cytochrome-c released.

These type of caspases and their activation domains are not reported in plant systems, even though cytochrome –c release was observed during plant PCD (Manon *et al*., 1997) and a number of R-genes having sequence similarity with *Apaf-1* are reported (Stein and Hansen, 1999). The mammalian mitochondria act

as a store of various pro-apoptotic factors like Cytochrome-c, Bax, Bak, Apaf-1 and anti-apoptotic factors like Bcl-2 and Bcl-XL members of the family. Evidence of similar Bcl-2 homologue are reported in tobacco leaf cells with antibodies (Dion *et al*., 1997) and expression of human Bcl-2, Bcl-XL and Ced-9 showed the suppression of cell death triggered by fungal pathogenic elicitors (Dickman *et al*., 2001), homologue of Human Bax inhibitor in *Arabidopsis* (AtBI-1) and Rice (OsBH-1) reported to suppresses the yeast cell death (Sanchez *et al*., 2000).

However, animal casapse like activities were detected in tobacco exposed to virus, (delpozo and Lam, 1998) and in tomato after chemical-induced apoptosis (de Jong *et al*., 2002), in *Arabidopsis* after nitric oxide-induced cell death (Clarke *et al*., 2000) and in embryonic suspension cells from barley (Korhtout *et al*., 2000) and these caspase-like activities could be inhibited with caspase inhibitors is also reported in plant systems.

Homology searches across the plant genome suggest that existence of metacasapses with casapase conserved domains in plants and fungi (Uren *et al*., 2000).

Recently, Hatsugai *et al.,* (2004) reported that a vacuole localized protease called VPE exhibits caspase-1-like activity and regulates cell death in both resistant and susceptible responses to pathogen infection. The role of VPE is explained in Fig 1.4.

2.3 Inducers of p+rogrammed cell death.

2.3.1 Plant programmed cell death inducers.

PCD research in plants was initially focused on identifying similarities with animal systems. However, several morphological and biochemical similarities are observed between animal apoptosis and plant PCD like condensation and shrinkage of the cytoplasm and nucleus, the formation of apoptotic bodies and

genomic DNA degradation (Danon *et al*., 2000).These characters are also observed in plants during senescence, endosperm development, HR response, tracheary elements differentiation and various forms of abiotic stresses (Danon *et al*., 2000).

External addition of the ROS member, H_2O_2 is reported to induce PCD in tobacco cells (Desikan *et al*., 1998), in *Arabidopsis* cells and protoplasts (Neill *et al*., 2002), in suspension cultures of soyabean (Levine *et al*.,1994). The other stimulants leading to accelerated PCD in plant systems are exposure to ozone, cold stress, UV-radiation and developmental phases like senescence, tracheary elements (TE) differentiation, aleurone layer breakdown during seed germination and leaf morphogenesis.

The most widely reported plant PCD stimulants are H_2O_2 , HR elicitors(Sasabe *et al*., 2000), heat shock(Tian *et al*.,2000), NaCl, sugar, salicylic acid(Xie *et al*., 2000), ethylene (Dejong *et al*., 2002), cryptogien , menadione (Sun *et al*., 1999), *Agrobacterium* infection (Hansen *et al*., 2000), harpin (Desikan *et al*., 1998), nitric oxide (Delldone *et al*., 2001), fuminosin(Asai, 2000), ABA in maize(Young and Galie, 2000). Rao and Davis (2001) demonstrated that treatment with salicylic acid (SA) causes PCD with enhanced levels of H_2O_2 production, lipid peroxidation and oxidative damage to proteins.

2.3.2 Yeast programmed cell death inducers

S.cerevisiae has been used to analyze the pro-apoptotic genes from mammalian, Drosophila and plant origin by assuming it is a clean room for apoptotic studies and to investigate interaction between the two gene products. Apoptotic cell death occurrence in yeast was demonstrated by expressing Bax/bak (Ink *et al*., 1997) in *S.pombe* with typical apoptotic markers like chromosome condensation and DNA cleavage. Ligr *et al., (1998)* expressed Bax in *S.cerevisiae*

and showed its death type by analyzing TUNNEL assay, Annexin-V staining and chromatin condensation.

In yeast, the mammalian pro-apoptotic (Bax, bak, ced-9) and anti-apoptotic (Bcl-2, Bcl-XL) genes are expressed and characterized. While expressing these genes, yeast phenotype was characterized to apoptotic characterstics (Ink *et al*., 1997). This important yeast system is also used to screen inhibitor of apoptosis against Bax mediated cell death. Initially, identification of yeast homologues to mammalian pro-apoptotic /anti-apoptotic proteins failed till Madeo *et al.,* (1997) showed *cdc48* mutant exhibits, typical hallmarks of apoptosis.

Madeo *et al.* (1997) report on cdc48 mutant with apoptotic characters triggered the search for yeast apoptotic stimuli. The various stress conditions or drugs reported to inducing apoptosis in yeast are low doses of H_2O_2 (Madeo *et al*., 1999), aspirin (Balzan *et al*., 2004), HOCl (King *et al*., 2004), acetic acid(Ludovico *et al*., 2002), hyperosmotic (Silva *et al*.,2005), sugar (Granot *et al*., 2003) and NaCl (Huh *et al*., 2002).

The DNA damaging drugs like adozelsin(Blanchard *et al*., 2002), amphotericin B(Philips *et al*., 2003), low levels of valproic acid(Mitsui *et al*., 2005), few viral killer toxins (Reiter *et al*., 2004), Uv radiations, cell ageing(Herker *et al*., 2004, Laun *et al.*, 2001) and pheromones are also reported to induce yeast PCD (Severin and Hyman, 2002).

2.4 Signal transduction during Programmed cell death

2.4.1 Plant PCD-signal transduction pathways

Plant hormones, ABA, GA, SA and ethylene are strong mediators of cell death. ABA and GA control cell death during development, while SA and ethylene are important for cell death during HR.SA activates cell death at higher concentrations and survival signals at lower concentrations, thereby may control

the extent of cell death during HR (Alvarez, 2000). Jasmonic acid (JA) inhibits cell death during defense and stress response (Overmyer *et al*., 2000; Rao and Davis, 2001). As a general mode of action, hormones may attenuate ROS level. Many studies have indicated central role of ROS in PCD (Fig 6).

2.4.2 ROS signaling in programmed cell death

Aerobic condition is directly linked with ROS production in living systems. In plants, ROS are generated as by-products during basic metabolic processes like photosynthesis and respiration with a complex enzyme machinery of oxidases in subcellular organelles like chloroplasts, peroxisomes, and mitochondria (Apel and Hirt, 2004). Even the presence of effective antioxidant machinery in these organelles, equilibrium changes in ROS homeostasis are observed and inevitable during abiotic and biotic stress tolerance (Foyer and Noctor, 2005), When the rise in ROS concentration levels are limited, the antioxidant capacity is sufficient to bring back the original balance between ROS production and scavenging to establish basic redox homeostasis.

Under abiotic and biotic environmental stress, this delicate redox balance is easily disturbed, resulting significant in ROS accumulation. Infact, a 3- to 10-fold increase in ROS levels has been calculated under stress conditions (Polle., 2001). Therefore, it is not surprising that a transient oxidative burst and a subsequent temporary shift in the intracellular redox state are common features of both biotic and abiotic stress responses (Dat *et al*., 2000; Mittler *et al*., 2004). Under unfavourable environmental conditions such as temperature extremes, drought, or salt stress, the rate of carbon fixation is limited, causing an increase in photoinhibition, potentially steering the photosystem toward overproduction of superoxide radicals and H_2O_2 (Foyer and Noctor, 2005). Even during ozone exposure, ROS are generated following the entry of ozone through the stomata and its conversion in the leaf apoplast, eventually leading to the formation of HR-like

lesions (Pellinen *et al*., 1999; Rao and Davis, 2001). Incompatible plant-pathogen interactions shows biphasic oxidative burst during which both infected and adjacent cells are sacrificed to limit further spreading of pathogen (Draper, 1997).

The production and accumulation of ROS in the apoplast occurs mainly from an increased activity of apoplastic peroxidases, amine oxidases, and an NADPH-oxidase complex coupled to a decrease in cellular ROS-scavenging capacity (Bolwell, 1999; Mittler *et al*., 1999; Torres and Dangl, 2002).

The resulting oxidative burst reactive oxygen species affect various cellular mechanisms like destructive protein modifications, mutagenic DNA strand breaks, purine oxidations and protein-DNA cross links. Lipid peroxidation in both cellular and organellar membranes takes place due to increase in ROS levels, which results in production of lipid-derived radicals. At least 158 genes in the *Arabidopsis* genome specifically master the spatial-temporal network of ROS production and scavenging (Mittler *et al*., 2004).

2.4.3 ROS signals involved in plant cell death

The involvement of ROS in PCD was established based on spatio-temporal correlations between increased ROS levels and cell death. The initial experimental evidence that ROS also act as a signal in plant PCD was obtained in cell suspensions by demonstrating H_2O_2-induced cell death could be blocked by cycloheximide and protease inhibitors (Levine *et al*., 1994). Since then, there has been ever growing support for a key role for ROS as triggers of PCD. This was demonstrated by exogenous application of oxidants and development of transgenic plants to modify the levels of cellular antioxidants and mutants which are unable to stop the initiation or propagation of ROS. (Mittler and Rizhsky, 2000; Lorrain *et al*., 2003)

The examples of ROS-derived PCD occurs during HR and ozone stress. The cell death is characterized by observation of several hallmarks of PCD, such as chromatin condensation, DNA laddering, and cytochrome-c release (Lam, 2004). These characters are halted by adding either high concentrations of antioxidants or inhibitors of translation, transcription and signal transduction components, such as kinases or phytohormones. Few transgenic plants with low or high levels of several antioxidants (superoxide dismutase, catalase, and ascorbate peroxidase) also exhibit an altered response to both pathogen- and ozone-driven PCD; this phenomenon clearly explains importance of regulated redox balance in plant system (Van Camp *et al*., 1998; Mittler *et al*., 1999). The ROS-dependent PCD pathway in plants is explained by using transgenic plants deficient in catalase (Cat1AS, the major H_2O_2-scavenging enzyme). In Cat1AS leaves of tobacco (*Nicotiana tabacum*), accumulation of H_2O_2 was observed and an active cell death in leaf palisade parenchyma cells was observed with several PCD hallmarks, this is inhibited by infiltration of various antagonists of HR-like PCD (Dat *et al*., 2000).

A number of *Arabidopsis* mutants have been used to demonstrate the specificity the signaling different ROS in initiating PCD. Two mutants of *Arabidopsis*, *lesion-stimulating disease1* (*lsd1*) and *radical-induced cell death1* (*rcd1*), elevated ROS levels are necessary and sufficient to induce spreading of cell death (Jabs *et al*., 1997; Overmyer *et al*., 2000; Madeo *et al*., 2004). The conditional *fluorescent* (*flu*) mutant of *Arabidopsis* that generates singlet oxygen upon a dark-to-light shift initiates a cell death response immediately after the release of singlet oxygen. The ozone-sensitive mutant (vitamin *c-1* (*vtc-1*) deficient in L-ascorbic acid exhibit HR like lesions (Pavet *et al*., 2005) These lesions resulted due to changes in redox homeostasis rather than to H_2O_2, because of lowering of ascorbate levels in *vtc* mutants to that of wild-type plants (Pavet *et*

al., 2005). In summary, various ROS species and cellular redox changes can trigger different signaling cascades leading to PCD.

2.4.4 ROS signal relay during plant cell death

In animal cells, mitochondria are recognized as central players in ROS-dependent apoptotic cell death. The important step during apoptosis is the release of cytochrome-c to activate caspase cascade, which precedes mitochondrial membrane depolarization and nuclear condensation as well as other hallmarks of apoptosis. In plant mitochondria, where the initial alteration in ROS homeostasis is amplified, triggering cytochrome-c release through mitochondrial transition pore opening and morphological changes (Tiwari *et al*., 2002; Dat *et al*., 2003; Casolo *et al.,* 2005) demonstrated by up-regulation of both manganese superoxide dismutase (MnSOD) and the alternative oxidase (AOX) mitochondrial antioxidant genes early during ROS-driven PCD (Robson and Vanlerberghe, 2002; Dat *et al*., 2000).

In plants system other than mitochondria, chloroplast is the other site to produce ROS. For example, during cryptogein-induced PCD, an H_2O_2-dependent activation of lipoxygenases target chloroplastic polyunsaturated fatty acids, releasing oxylipins that are sufficient to trigger PCD (Rustérucci *et al*., 1996; Maccarrone *et al*., 2000; Montillet *et al*., 2005) and chloroplasts involvement are further demonstrated by ectopic expression of mammalian antiapoptotic B-cell leukemia/lymphoma (BCL2) members in the chloroplasts, which protect transgenic tobacco plants from herbicide-induced PCD (Chen and Dickman, 2004).

Finally, in addition to mitochondria and chloroplasts, a strong interplay between ROS and other signaling molecules (phytohormones) are reported during plant PCD (Overmyer *et al*., 2005). The ROS-dependent PCD is associated with increased levels of both salicylic acid (SA) and ethylene, which strongly positions

both ethylene and SA promotion of ROS-dependent cell death (de Jong *et al*., 2002; Moeder *et al*., 2002) Another phytohormone, jasmonic acid can either induce or act antagonistically in this oxidative-dependent cell death cycle depending on the initial ROS signal is singlet oxygen or superoxide (Overmyer *et al*., 2000, Danon *et al*., 2000).

Therefore Redox-dependent plant PCD involves a range of signaling molecules, and an interactive regulatory network illustrated in Fig. 6.

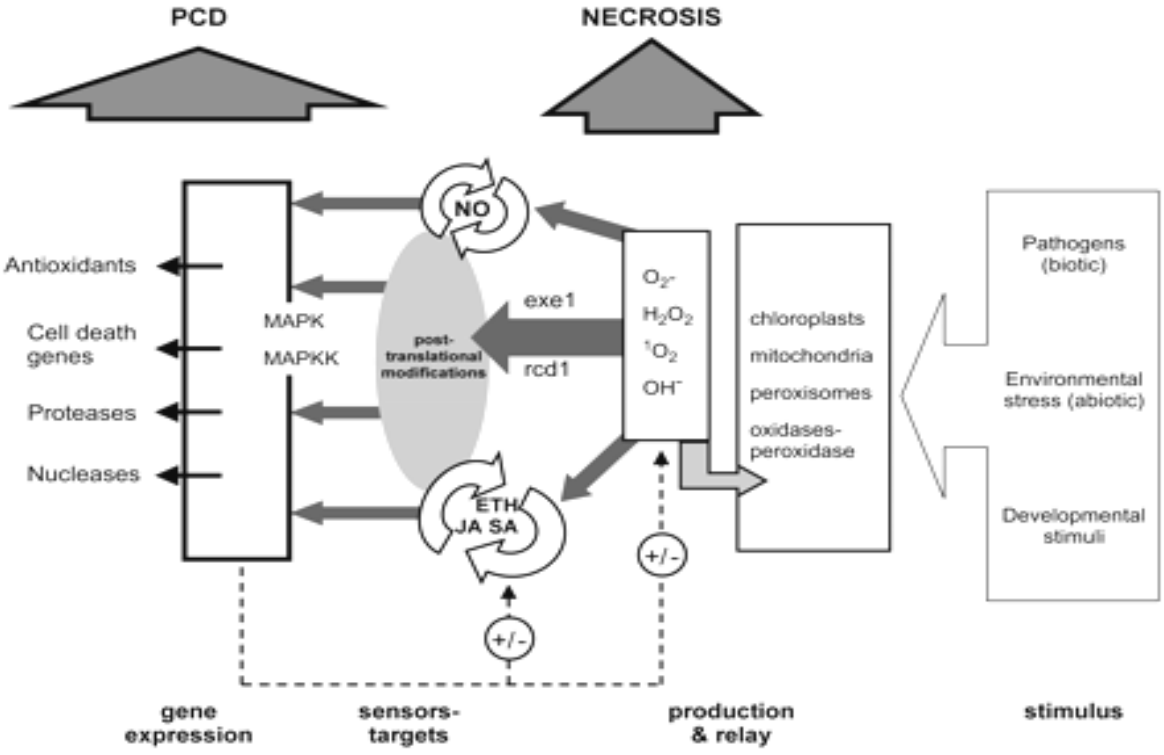


Figure 6: Schematic representation of ROS-dependent cell death pathways.

2.5 Molecular targets of ROS signaling during plant cell death

The first gene identified in ROS-dependent cell death is *Arabidopsis lsd1,* the mutant of this gene is sensitive to death with increased superoxide levels and develops spontaneous superoxide- and SA-dependent runaway lesions (Jabs *et al*., 1997). Later LSD1 identified as a sensor molecule, which senses the changes in ROS homeostasis, thereby repressing a default death pathway or controlling an anti-death mechanism through the regulation of cell death genes (Dietrich *et al*., 1997; Epple *et al*., 2003). Another ozone-sensitive lesion mimic mutant, *rcd1*, also displays PCD lesions. RCD1 mainly modulates the activity of target proteins through ADP ribosylation (Ahlfors *et al*., 2004; Overmyer *et al*., 2005). Recently, Wagner *et al*. (2004) showed ROS-induced PCD not to be mediated by photooxidative damage but through a genetically determined PCD pathway by using inactivated *Arabidopsis Executer1* gene, which is resistant to singlet oxygen-induced cell death.

Other than what the mentioned above, another class of signal transducers like mitogen-activated protein kinases (MAPKs) act upstream of the oxidative burst during ozone treatment and the HR (Ren *et al*., 2002; Samuel and Ellis, 2002). The primary ROS-activated tobacco MAPK is the SA-induced protein kinase, which is required during harpin-dependent PCD (Samuel *et al*., 2005). A MAPKKK of alfalfa (*Medicago sativa*) activates cell death induced by H_2O_2 through a specific MAPK-scaffolding action (Nakagami *et al*., 2004). Overlapping features of animal and plant PCD have also inspired different functional approaches by introducing animal genes into plants to gather mechanistic insight into oxidative stress-dependent cell death events. No obvious homologs of the BCL-like animal cell death suppressors have been identified in plant genomes to date; nevertheless, protection against mitochondrial and chloroplast-derived ROS-dependent cell death is conferred by their overproduction in tobacco. Functional plant homologs been successful in protecting against ROS-mediated PCD in the

transgenic lines, their existence remains unclear (Mitsuhara *et al*., 1999; Chen and Dickman, 2004).

2.5.1 Calcium signaling in PCD

The calcium signature hypothesis has evolved as a concept to explain specificity in signaling pathways that utilize calcium as a second messenger. In this hypothesis, a fine tuning of calcium homeostasis by regulation of intracellular and extracellular stores calcium release and sequestration is important for both survival and death in biological systems. The apoptosis/ PCD known form of active cell demise occurs due to loss in Ca^{2+} homeostasis in a cell. Later, evidence is establishment of link between DNA fragmentation pattern in isolated lymphocyte nuclei with activation of Ca^{2+} and Mg^{++} dependent endonuclease (Wylie *et al*.,1980; Cohen *et al*., 1994). Subsequently extracellular Ca^{2+} is necessary in a glucocorticoid –induced apoptosis in lymphocytes. Recently (Inositol (1,4,5)P3 receptor up regulation in lymphocytes, which are induced either by IgM or dexamethasone provide evidences to a need of Ca^{2+} in lymphocyte apoptosis.

Elevation of $(Ca2^{+})_{Cyt}$ have been recorded in response to a wide range of environmental , developmental and growth stimuli in an equally wide range of tissues and cell types. In plant PCD, calcium has also been reported as a ubiquitous signal and its rise reported during tracherary elements differentiation, aerenchyma formation (Drew and Morgan. 2000), wheat aleurone differentiation, the HR (Levine *et al*., 1996) and leaf senescence. The plasma membrane Ca^{2+} blockers, Lanthnum chloride inhibit H_2O_2 induced apoptosis cell death in soybean cells, bacteria –induced PCD in *A.thaliana* and camptothecin –induced PCD in tomato cells.

2.5.2 $Ca2^{+}$ signaling in yeast

In eukaryotic cells, the Ca^{2+} functions as a ubiquitous intracellular messenger by which extracellular factors induce a variety of physiological responses such as proliferation and muscle contradiction.

The cytosolic calcium concentration is in the range of 50-200nm in normal yeast cells (*S.cerevisiae*). When yeast cells are exposed to environmental abiotic and biotic factors, Ca^{2+} will raise upto 1um to 100mM. The calcium homeostasis in a yeast cell involves the regulation of internalization and sequestration of Calcium into a variety of intracellular compartments such as ER, Golgi apparatus through many membrane proteins. The rise of cytosolic calcium is also observed during pheromone stimulation (Muller *et al*., 2001), hypertonic shock (Batzia *et al*., 1996) and NaCl stresses (Matsumato *et al*., 2002).

2.6 Nitric oxide as a signal molecule

Nitrogen monoxide or nitric oxide (NO) was discovered by Joseph Priestley in 1772. The free radical NO has generated profound interest over a period of last 20 years. In 1980, Furchgott and Zawadzki reported that endothelial cells released a substance which is responsible for the relaxation of vascular smooth muscle (they called this substance “endothelium-derived relaxing factor”: EDRF). In 1992, *Science* named NO “Molecule of the Year” because of its widespread biological significance (Koshland, 1992). Robert F. Furchgott, Louis J. Ignarro and Ferid Murad received Nobel Prize in Physiology and Medicine in 1998 for their contribution concerning "nitric oxide as a signaling molecule in the cardiovascular system". The NO functions in biological systems can be grouped into two basic categories, i) direct effects and ii) indirect effects, based on the concentration of NO. Where high and sustained amounts of NO are produced, the indirect effects are observed in those sites. The non-enzymatic interaction of NO with oxygen and superoxide (O_2^-) generates reactive nitrogen species and ROS,

before initiation of chemical modification of biological targets. Direct effects are the ones in which NO reacts directly with metal complexes (*e.g.* heme, guanylate cyclase (GC)), non- heme iron proteins, zinc and copper proteins and powerful free radicals. These reactions require low concentrations of NO (< 1 μM).

The reaction between NO and O_2^-, in gas phase and in water solution, generates $ONOO^-$ (peroxynitrite) (Figure 5). The half-life of $ONOO^-$ is of the order of one second under physiological conditions. At physiological pH, $ONOO^-$ equilibrates rapidly with peroxynitrous acid (ONOOH), which spontaneously decomposes to NO_3^- or to the highly reactive hydroxyl radical $HO^\bullet$ Even $ONOO^-$ is a potent and versatile oxidant capable of initiating lipid peroxidation and oxidizing thiols or lipid soluble antioxidants and it also react with thiols (S-nitrosation) to produce S-nitrosothiols (RSNO) (Figure 5).

NO reduces total cell respiration and inhibits ATP synthesis (oxidative phosphorylation) by inhibition of the cytochrome pathway (Yamasaki *et al*., 2001), which favors electron flow through the alternative oxidase pathway (Zottini *et al*., 2002). NO also interacts with key enzymes that participate in early defense, like catalase and ascorbate peroxidase (Clark *et al*., 2000).

2.6.1 Functions of Nitric Oxide in plants

2.6.2 NO during the plant life cycle: growth and development

Plants emit NO under normal growing conditions and NO and its related nitrogen dioxides have been reported as stimulators of seed germination (Grubisic and Konjevic, 1992). Detrimental effects of NO on photosynthesis was reported and subsequently the effects of NO on plant growth were found to be concentration dependent (Hufton *et al*., 1996; Leshem and Haramaty, 1996; Saviani *et al*., 2002). It was reported that NO can reversibly suppress electron transport and ATP synthesis in chloroplasts (Takahashi and Yamasaki,

2002).Morot-Gaudry-Talarmain *et al*. (2002) have shown that antisense-NiR tobacco plants accumulate nitrite, emit large amounts of NO and exhibit reduced growth. In the "pea disc expansion assay", low NO levels promote foliage expansion growth, while higher levels are increasingly inhibitory (Leshem *et al*., 1998).

Recently, it has been reported that in *Atnos*1 mutant plants (*Arabidopsis* mutant plants with impaired NO production) treated with 100 μM SNP, greening, growth, and fertility were restored, indicating that these phenotypes were due to a deficiency in NO production caused by the *Atnos*1 mutation (Guo *et al*., 2003). The positive effects of NO on chlorophyll retention may reflect NO effects on iron availability. NO is closely related to iron metabolism (Murgia *et al*., 2002), transport, and/or availability and, consequently, to chlorophyll biosynthesis and chloroplast development Working with different plants.

2.6.3 Nitric Oxide in plant pathology

In nature, plants continuously defend themselves against attack from abiotic and biotic challenges. Therefore, each plant cell possesses both preformed and an inducible defense system. One of the most powerful weapons in plant's arsenal against pathogen attack is the hypersensitive response (HR), which could be considered as a programmed cell death (PCD) (Jabs *et al*., 1997). The HR is characterized by rapid, localized cell death at the site of infection (Lamb and Dixon, 1997) and it plays a central role in disease resistance (Heath, 2000). A localalised HR is often associated with the onset of systemic acquired resistance (SAR) in distal plant tissues (Enyedi *et al.,* 1992; Ryals *et al.,* 1996).

Although the effects of both ROS (H_2O_2) and NO on plant physiology and development have been the subject of investigation for several years, it is only

relatively less known about their role as signaling molecules during abiotic or biotic stresses (Neill *et al*., 2002b).

2.6.4 Nitric Oxide in HR and programmed cell death in plants.

Pathogen attacks cause very localized NO production and localized high NO concentrations. This NO appears as a second messenger in plant pathogen resistance (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Bolwell, 1999; Clarke *et al.*, 2000). NO donors themselves were able to provoke a HR (Huang and Knopp, 1998) and induce cell death in *Arabidopsis* suspension culture when present at concentrations similar to those generated following challenge by avirulent pathogens (Clarke *et al*., 2000). Saviani *et al*. (2002) reported that treatment of citrus cultures with NO donors causes cell death bearing the characteristics of PCD. Soybean and *Arabidopsis* cell suspensions inoculated with *Pseudomonas syringae* produce NO with a pattern similar to H_2O_2 accumulation (Delledonne *et al*., 1998; Clarke *et al*., 2000). In this case, an initial rapid, but transient, stimulation of NO accumulation is induced by both avirulent and virulent *Pseudomonas syringae* strains. However, this is followed by sustained production of NO, only in the cells inoculated with the avirulent strain. NOS activity was detected in this plant-pathogen interaction (Delledonne *et al*., 1998). With immunoblot analysis using antibodies against rabbit brain NOS; it has been found that tobacco plants resistant to infection by *Ralstonia solacearum* exhibited elevated levels of NOS. Recently, Chandok *et al*., (2003) demonstrated that the pathogen-inducible NOS (iNOS) in plants is a variant form of the P protein of GDC. Addition of a membrane permeable NO scavenger or NOS inhibitors completely blocked NO-dependent cell death and defense gene activation in tobacco and soybean (Delledonne *et al*., 1998; Durner *et al*., 1998), showing that NO plays a role in HR development.

PCD occurring as a result of mechanical stress also involve NO. In *Kalanchoe daigremontiana,* centrifugation of leaves and callus induced NO generation and subsequent DNA fragmentation and cell death (Pedroso and Durzan, 2000; Pedroso *et al*., 2000). Decreased NO synthesis and PCD in the presence of a NOS inhibitors suggested the involvement of a NOS-like enzyme in this species. Similar results were found with mechanical stress of *Arabidopsis* tissues.

2.7 Sub cellular organelles involved in programmed cell death

2.7.1 Role of mitochondria in programmed cell-death

Mitochondrion is the power house of the cell, involved in the production of biological currency in the form of ATP. The mitochondrial roles in programmed cell death pathways were reported in animal systems, where its involvement in PCD in plant has been suggested by Jones (2001) and then, in particular, related to the manifestation of HR (Lam *et al*., 2001). The key role of mitochondria is more than just a `loss of function' resulting in an energy deficit, but is an active process involving different mitochondrial proteins (pro and anti-apoptotic) release and further downstream process (Green and Kroemer, 2004; Kroemer and Reed, 2000). Importantly, Cytochrome-c was the first mitochondrial factor shown to be released from the mitochondrial intermembrane space and its involvement in apoptotic cell death. Since then, other mitochondrial proteins, such as AIF, Smac/DIABLO, endonuclease G and Omi/HtrA2, were found to undergo release during apoptosis and have been implicated in various aspects of the cell death process. Members of the Bcl-2 protein family Bcl family proteins (anti apoptotic-Bcl-2, Bcl-Xl, pro-apoptotic Bax, Bak, Bid, Bad) control the integrity and response of mitochondria to apoptotic signals. The molecular mechanism by which mitochondrial inter-membrane space proteins get released and the regulation of mitochondrial homeostasis by Bcl-2 proteins is still elusive.

The involvement of mitochondria and its constituents in the regulation of apoptotic cell death was first described in terms of disruption/loss of the mitochondrial transmembrane potential (MMP), MMP collapse was considered as the `point-of-no-return' of the death program (Kroemer *et al*, 1995). The important evidence against central role of mitochondria in apoptosis came with cytochrome c, an electron shuttle molecule in the oxidative phosphorylation pathway and involvement in Caspase activation after its release from mitochondria.

In vitro studies have shown that proteins associate with and modify the permeability of the outer mitochondrial membrane (OMM). This is thought to reflect the ability of these proteins to form channels, or to interact with and modify other proteins that form ion conducting channels (for example, by promoting or inhibiting oligomerization of channel subunits). In addition to responding to proteinaceous intracellular death signals, the mitochondrion may also initiate apoptosis in response to changes in the levels of cellular messengers such as calcium, to changes in cellular pH or to changes in the levels of metabolites that reflect the energy status of the cell (for example, ATP, ADP, NADH, NADPH and creatine phosphate), (Vander.H *et al*., 2002). Increased permeability of the OMM may lead to the release from the mitochondrion of a number of cell-death activators, inhibitors and inhibitor de-repressors, including cytochrome *c*, apoptosis-inducing factor (AIF) and Smac/DIABLO (Fig. 2). Among these proteins, cytochrome *c* has been shown to regulate the activity of the initiator Caspase, procaspase-9, by means of the adaptor protein Apaf-1. AIF, another executor of PCD released from the mitochondrion, is a large protein that can enter the nucleus and activate the fragmentation of chromatin DNA to 50-kilobase-pair fragments (Susin *et al*., 1998).

Therefore, mitochondrion functions as an important storehouse for key cell death-signalling proteins, which are kept physically sequestered from their targets

until the appropriate execution order has been given. Disruption of the OMM and leakage of cytochrome *c* to the cytosol may also lead to inhibition of electron flow from complex III to complex IV in the inner mitochondrial membrane (IMM). This in turn may lead to generation of reactive oxygen species (ROS) that can serve as amplification signals for PCD (Balk *et al*., 1999).

Several lines of evidence point towards the importance of the mitochondrion in the expression of HR-associated PCD in plants. At present, it is not clear whether cytochrome-c leakage also occurs during the HR, although leakage has been observed in plant cells undergoing PCD in response to other (non-pathogenic) inducers (Lam *et al*, 1999). Bacterial virulence factor harpin disrupts mitochondrial functions (Xie and Chen, 1999). The expressed Bax protein in the mitochondria results in PCD, whereas mutations that affect its oligomerization suppress this activity. These observations suggest that Bax activates cell death in plants using mechanisms that are fundamentally similar to those present in animal cells. The involvement of plant mitochondria in HR-associated cell death comes from studies of the alternative oxidase (AOX), an IMM enzyme that is not found in animal mitochondria. (Chivasa and Carr, 1998). Antisense suppression of AOX resulted in hypersensitivity to antimycin A, overexpression of AOX has the reverse effects, providing supporting evidence for a model in which plant mitochondria have an important role as a signal generator for HR-induced cell death.

The above described mitochondrial apoptotic factors are still not reported in plants systems. The most common mitochondrial hall mark in plant PCD is the release of cytochrome-c (observed in the cytosol of carrot cells and later which is prevented by Caspase inhibitors (Zhao *et al*., 2001)).The release of Cytochrome –c has been reported in many plant systems, In particular, mannose induced cell death in *Arabdopsis thaliana* (Stein and Hansen, 1999) and in maize cells infected with

Agrobacterium sps(Hansen, 2000), harpin induced PCD in tobacco cells(Xie and Chen, 2000).Induction of PCD in A.thaliana cell cultures by ceramide, protoporphyrin IX and an elicitor of HR leads to the dissipation of $\Delta\psi M$, morphological changes and cytochrome -c release.. But no Cytochrome -c release was observed during petal senescence.

2.7.2 Role of vacuole in Programmed cell death

The presence of apoptotic bodies and DNA laddering are two key characteristics of an apoptotic or programmed cell death and these characters are observed at defined time points. The ability to make these decisions is especially relevant to plant cells, because cell content management processing in plants is autolytic and plants lack macrophages and neutrophil cells to make these decisions for them. The cytological characters of cell death during hypersensitive response (HR), terminal differentiation and senescence revealed the action of the vacuole to degrade the cellular contents.

In 1979, Boller and Kende confirmed Philippe Matile's hypothesis that the plant cell vacuole is a large hydrolytic compartment and versatile organelle, vacuole played a primary role in PCD of the developing tracheary element and it was clearly shown by Groover and Jones (1999), through video microscopy, that the collapse of the vacuole coincides with cessation of cytoplasmic streaming and that this collapse marks the onset of autolysis during TE differentiation.

The vacuole collapse often releases hydrolases, which effectively act as a large suicide by calcium flux. Vacuole collapse and chromatin degradation revealed by TUNEL (TdT-mediated dUTP nick-and labeling) analysis turned out to be such robust markers of PCD in these cells (i.e. these markers confidently distinguish necrosis from PCD.

2.7.3 Cell content digestion and removal by vacuole proteases during programmed cell death

It appears that collapse of the vacuole may be the universal trigger of plant cell death; however, the differences in the way death is manifested results from different mechanisms for processing the cell corpse. Figure 4 illustrates a model for death and corpse processing that incorporates the similarities and differences between three plant PCD types.

Metabolically active hydrolases are synthesized and sequestered into the vacuole and releases during vacuole collapses. Based on the integration of signals, the cell creates a profile of hydrolases in the vacuole that establishes the way the corpse is processed. For example, auxin and cytokinin induce the *de novo* synthesis of vacuole-sequestered nucleases and proteases but obviously not the hydrolytic activity that would remove the secondary wall that a tracheary element builds prior to its death.

In contrast, during lysigenous aerenchyma formation induced by ethylene, cell wall hydrolases such as cellulase are included to fulfill the need to remove not only the protoplasm but the extracellular matrix as well. In the hypersensitive response, signals from pathogens in most cases induce the production of toxic phytoalexins, polyphenols, and chitinases, and these are released when the vacuole collapses.

Cell death is triggered after the cell has prepared to carry out hydrolytic events. This component of plant PCD may be shared by all or most forms to date, namely the \ vacuole collapse mediated by a calcium flux. Release of the vacuole contents marks the beginning of the removal of cell contents. Like during aerenchyma formation, complete hydrolysis of the cell results, whereas tracheary elements differentiation, remove only the protoplasm. Death in the hypersensitive

response does not remove the cell contents, but releases toxins directed against the pathogen.

2.7.4 Perxisomes and programmed cell death

Peroxisomes, are small (about 0.1 - 1.0 mM in diameter), ubiquitous subcellular organelles found in both animal and plant cells. Peroxisomes contain catalase and hydrogen peroxide-producing oxidases like fatty acyl-CoA oxidase, Oxidases involved in break down of organic molecules by the process of oxidation to produce hydrogen peroxide, which is then quickly converted to oxygen and water by catalase. In plants, peroxisomes present in germinating seeds convert fatty acids and lipids to sugars for metabolism. Peroxisomes perform a number of complementary and auxiliary reactions in general cell metabolism, in particular the catabolism and anabolism of certain lipids, and therefore deserve consideration

The dominant position among oxido-reduction processes in peroxisomes is ascribed to catalase, a number of aerobic oxidases, and Cu,Zn-superoxide dismutase. The peroxidase reaction of catalase requires substrates for hydrogen donation, other than H_2O_2, e.g. alcohols, aldehydes, and formic acid.

III. MATERIAL AND METHODS

3.1 Material and methods (Tobacco Bright Yellow-2 cells)

3.1.1 Culture and maintenance of tobacco BY-2 cell line:

The tobacco Bright-Yellow-2 cell line was cultured and maintained as described by Nagata *et al.* (1992). Briefly, the culture was grown in a sterile liquid Murashige and Skoog(MS) medium supplemented with 3% sucrose, 0.2g KH_2PO_4, 100mg myoinositol, 1mg thiamine-HCl and 0.2mg 2,4-Dichlorophenoxy acetic acid (2,4-D) per liter of MS media. The suspension culture was grown in complete dark condition at 27 ^{o} C with constant shaking at 130rpm. The culture was maintained by sub culturing for every five days. 5ml of 5 days old culture was transferred to a fresh 50ml sterile MS-Medium and maintained for a period of 5 days. The cells were regularly checked under microscope (at 10X or 20X) to rule out any microbial contamination.

3.1.2 Synchronization of BY-2 cells:

15ml of stationary stage culture was transferred to 100ml of fresh sterile MS-medium supplemented with 5mg/L aphidicolin (Sigma) and incubated at 29^{o}C under complete dark for 24hrs with constant shaking (130rpm).The cells were washed extensively with fresh sterile MS medium (without 2,4-D and Thiamine HCl) and the washed cells were subjected to various treatments and observed under microscope.

3.1.3 Treatments

a) Hydrogen peroxide treatment

Cells in the stationary stage were exposed to 1mm, 5mM, 10mM and 15mM concentrations of H_2O_2 and control cells were treated with equal volume of sterile distilled water. 1ml of treated cells were collected at 0 to 6hr time intervals

and the percentage of viability of cells was determined by staining with 0.05% final concentration of Evans blue for about 10 minutes. The stained cells were washed with phosphate buffer saline (PBS, pH 7.4) and observed under bright field microscope with 40X and minimum 500 cells were scored for death parameters. The dead cells will appear in blue and live cells will not take up any stain. The stained cells were looked for cytoplasm shrinkage and vacuole collapse. 50µl of treated cells were dropped out on to a solid sterile MS medium at specific time intervals and plates were incubated for about 8 days to observe the growth parameters and were documented.

b) Heat treatment

The stationary cells were subjected to heat treatments viz 45^{o}C, 55^{o}C and 65^{o}C for 10 minutes duration and equal volume of control cells were maintained at 29^{o}c for 10 minutes. After heat shock, the cells were brought back to the 29^{o}C and incubated in complete dark with constant shaking (130rpm). 1ml of treated cells were collected at 0 to 6hr time intervals and the percentage of viability of cells was determined by staining with 0.05% final concentration of Evans blue for about 10 minutes. The stained cells were washed with phosphate buffer saline (PBS, pH 7.4) and observed under bright field microscope with 40X and minimum 500 cells were scored for death cells and the treated cells were dropped out as described earlier.

c) Sodium chloride treatment

BY-2 cells in the stationary phase were exposed to 100mM, 200mM, 400mM, 800mM and 1.0M NaCl and the control cells were treated by adding equal volume of sterile distilled water. The treated cells were incubated at 29^{o}C under dark condition with constant shaking (130rpm). 1ml of treated cells were collected at 0 to 6hr time intervals and the percentage of viability of cells was determined by staining with 0.05% final concentration of Evans blue for about 10

minutes. The stained cells were washed with phosphate buffer saline (PBS, pH 7.4) and observed under bright field microscope with 40X and minimum 500 cells were counted for death v/s live cells as previously stated and treated cells were dropped out as described earlier.

d) Mannitol treatment

BY-2 cell suspensions in the stationary phase were exposed to 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% mannitol. The control was maintained by adding sterile distilled water. Treated and control suspension cultures were incubated at 29^{o}C, under dark with constant shaking (130rpm). 1ml of treated cells were collected at 0 to 6hr time intervals and the percentage of viability of cells were determined and treated cells were dropped out as described earlier.

3.1.4 Analysis of treated cells for apoptotic markers/ features

a) Chromosomal DNA condensation and fragmentation

The Chromosome condensation and fragmentation is one of the key features in apoptotic or programmed cell death cellular process. This clearly explains that the cell under investigation is undergoing programmed cell death against specific stimuli. This character was monitored by staining the cells with DNA specific fluorescent dye like DAPI or Hoechst 100.

The different stimuli treated cells were collected at mentioned time intervals and fixed in 3.7% formaldehyde and 0.01% Triton-X-100 final concentration and stored at –20^{o}C temperature. These cells were washed with PBS, pH 7.4 and stained with 1ug/ml DAPI (Molecular probes) for about 30 minutes at 29^{o}C. The DAPI stained cells were washed with PBS (pH 7.4) to remove excess unstained DAPI. Then cells were visualized under Olympus Epiflourscence microscope with excitation (340nm) and emission filter (400nm) for DNA condensation and fragmentation.

b) Analysis of mitochondrial membrane potential collapse

Mitochondria are popularly known as the "powerhouses of the cell". It is a lipid bilayer subcellular organelle. It mediates a large number of important physiological processes like electron transport chain (ETC), cytochrome–c Mediated programmed cell death etc. in animals, plants and yeast. The loss of mitochondrial membrane potential (MMP-$\Delta\psi_m$) is an early event and it was clearly established in animal cells and plants cells when subjected to few stimuli. This aspect was also looked in tobacco BY-2 cell lines by using $\Delta\psi_m$ sensitive dye Rhodamine123. The stationary stage cells were preloaded with 100ng/ml of Rhodamine123 (Rh123) (Molecular probes) and incubated at 27^oC for about 30 minutes. Then the cells were washed three- four times with sterile MS medium to remove excess Rhodamine123.

These preloaded cells were subjected to above-mentioned stimuli (H_2O_2, NaCl, $CaCl_2$, salicylic acid, heat, mannitol) to analyze the loss of $\Delta\psi_m$ (MMP) by observing under epifluorescence microscope with excitation filter (304nm) and emission filter (551nm). The intact mitochondria were observed as a green dot like structure (The intact mitochondria will readily sequester Rh123), where as the MMP lost, or depolarized mitochondria will lose sequestered Rh123, thus, the entire cytosol will appear green.

c) Analysis of vacuole collapse against apoptotic stimuli

The different stimuli treated cells were collected at different time intervals and stained with 0.01% of Neutral Red. (The Neutral red enters into the live cells and sequester into a acidic compartment like vacuoles). The neutral red stained cells were observed under bright field microscope. Rupture of vacuole was clearly observed in the dead cells.

3.1.5 Analysis of protoplasts

a) Isolation of protoplasts from BY-2 cells

The 3 days old stationary phase cells were washed with protoplast buffer (0.5M mannitol, 20mM KCl, 3mM KH_2PO_4, 5mM $CaCl_2$, 1% sucrose, pH 5.2) and then incubated with digestion buffer (Protoplast buffer with 1.5% cellulase (W/V, Sigma) and 0.2% Pectinase (w/v, CalBiochem) for about 3-4 hours at 27^oC. Later released protoplasts were collected by centrifugation at 360g, for 5 minutes at 25^oC. Carefully the pellet was resupended in protoplast buffer and passed through mira cloth (to remove debris) and viability of the isolated protoplasts were determined by staining with Neutral Red.

b) Propidium Iodide(PI) staining- Loss of plasma membrane permeability analysis

The stressed tobacco protoplast were centrifuged at 360g at room temperature for 1 minute and incubated in protoplast buffer containing 1ug/ml PI for 10 minutes in dark condition. PI stained protoplasts were observed under Carl-Zeiss Apotome microscope and documented.

c) Measurement of total ROS

Several Reactive Oxygen Species (ROS)) are constantly produced in plants during their exposure to various biotic and abiotic stresses. Depending on the nature of the ROS species, the toxicity and further down stream processes will occur. But ROS species are detoxified by various cellular enzymatic mechanisms like SOD, APX etc. In plants, ROS is a signal molecule to control various processes including pathogen defense, programmed cell death and stomatal behavior. The ROS mainly activates the signal system through MAPK cascade through transcription or directly affecting gene expression.

Intracellular ROS production in BY-2 cells was monitored by using H_2DFCDA (Molecular probes) as a probe. In the presence of ROS, the non-colored probes get converted into a green colored product in cytosol. This can be detected under Epi-fluorescence microscope with an excitation filter of 450 to 490nm and a emission barrier filter 510nm.The cells were preloaded with 100nM/ml H_2DFCDA and stressed against different stimuli and immediately observed under Epi-fluorescence microscope.

3.1.6 Transformation of tobacco Bright yellow-2 cell lines with Atvam 5-GFP, At-BI-GFP, Fimbrin-GFPs and Actin-GFP by Balistic/Gene gun method as described by *Iida et al*, (1991)

Briefly, cells of 3 day old BY-2 cells were bombarded with tungsten particles (1.0µm) coated with above said constructs using particle–delivery system(PDS-1000/He, Bio-Rad, Hercules, CA,USA) according to the manufactures recommendations. BY-2 cells were placed at the distance of 6cm under the stopping screen and bombarded in a vaccum of 28 inches Hg at a Helium pressure of 1100Psi and bombarded cells were kept in the dark at 29^{o}C for 12hrs and then transferred to a selection medium and observed under confocal microscope. (Complete procedure in Appendix-II).

3.2 Materials and Methods (Yeast)

The budding yeast *Saccharomyces cerevisiae* is a unicellular, eukaryotic organism widely used to understand various basic physiological processes. The similarity between yeast and mammalian system in terms of subcellular structure and metabolic systems is almost unique. Therefore, budding yeast is used as one of the best model system in various laboratories to understand a basic process like respiration to highly complex process like development. We have also used the unique yeast model system to conduct the programmed cell death studies.

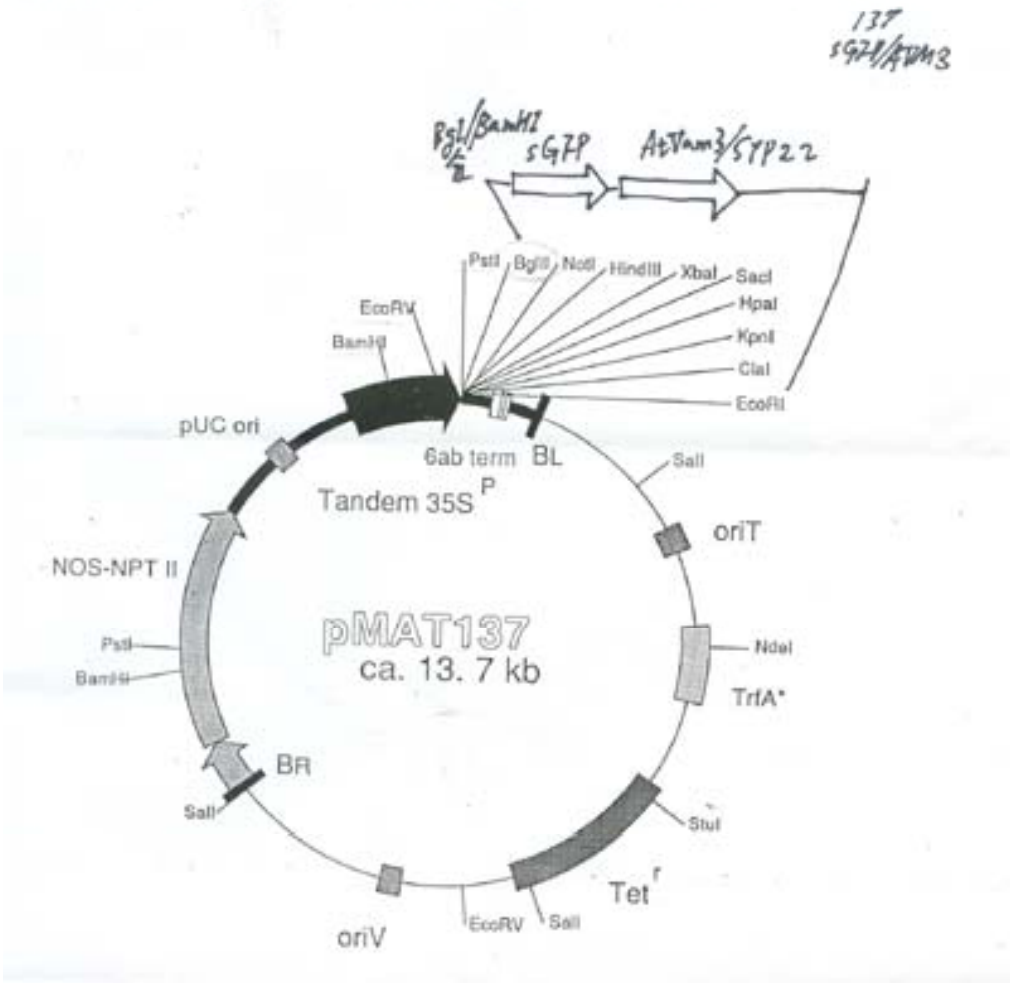


Fig. 7. Construct map of At vam5-GFP

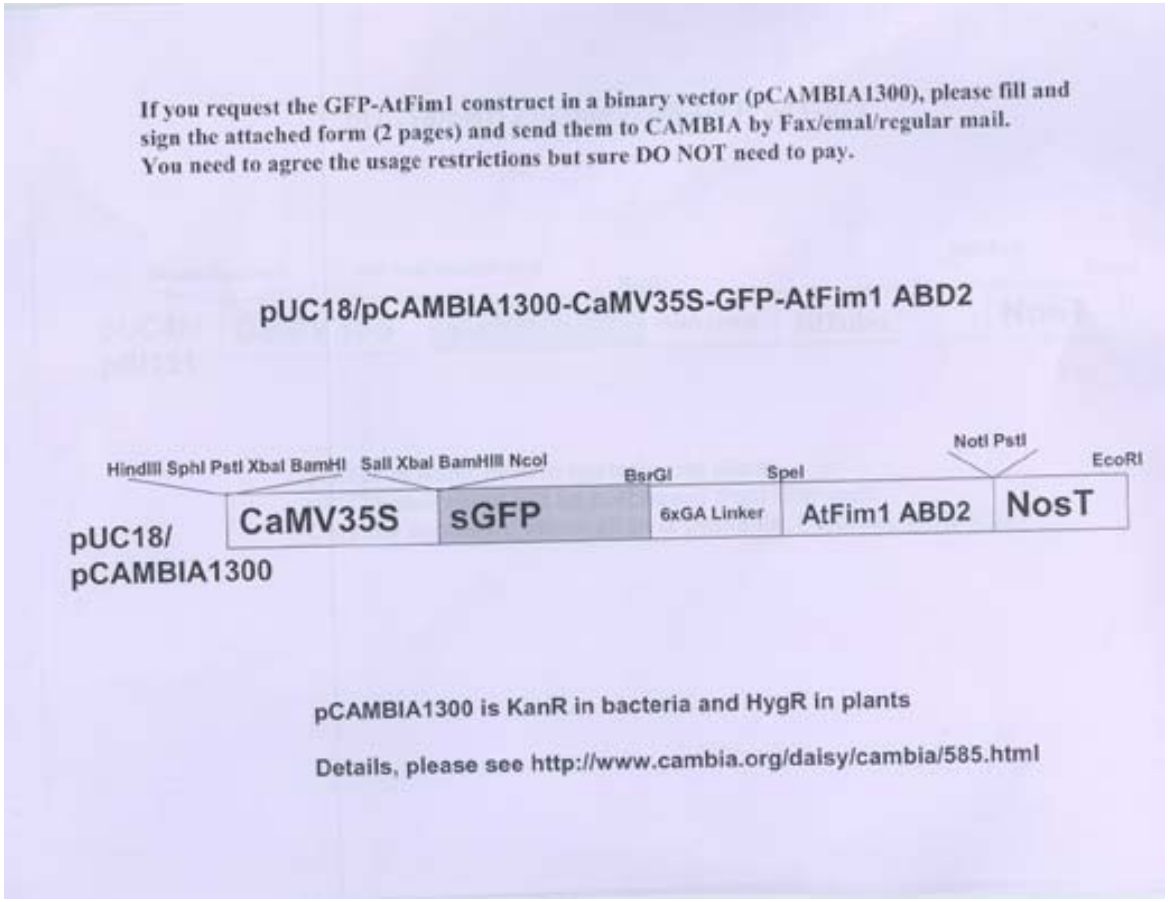


Fig. 8. Construct map of At Fim1-GFP

3.2.1 Yeast culture growth conditions

Yeast strain YPH-500 was grown aerobically in yeast peptone dextrose (YPD) medium, pH 5.5 to obtain an O.D of 0.5 at 600nm (stationary phase cells). The cells were subjected to various treatments (abiotic stresses)

3.2.2 Treatments

a) Hydrogen peroxide treatment

Cells in the stationary stage were exposed to various concentrations of H_2O_2 *viz* 1mm, 5mM, 10mM and 15mM H_2O_2 and control cells were also treated with equal volume of sterile distilled water. After H_2O_2 treatment the cells were brought back to 30^oC and were incubated with constant shaking. 1ml of treated cells was collected at 0 - 24hr with 6 hours time intervals and the viability of cells was determined by taking OD_{600}nm and growth curves were constructed.

b) Heat treatment

The stationary phase cells were subjected to heat treatments *viz* 45^oC, 55^oC and 65^oC, for 10 minutes and equal volume of control cells were maintained at 27^oC for 10 minutes. After heat shock, the cells were brought back to 30^oC and were incubated with constant shaking. 1ml of treated cells was collected at 0 - 24hr with 6 hours time intervals and the viability of cells was determined by taking OD at 600 nm and growth curves were constructed.

c) Sodium chloride treatment

Cells in the stationary phase were exposed to 0.5M, 1.0M, 1.5M, 2.0M and 2.5M, 3.0M NaCl and the control cells were treated by adding equal volume of sterile distilled water. The treated cells were incubated at 30^oC with constant shaking. 1ml of treated cells was collected at 0 - 24hr with 6 hours time interval

and the viability of cells was determined by taking OD at 600nm and growth curves were constructed.

d) Mannitol treatment

Cells in the stationary phase were exposed to 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% mannitol. The control was maintained by adding sterile distilled water. After the treatment, the suspension cultures were incubated at 30^{o}C, with constant shaking. The 1ml of treated cells was collected at 0 - 24hr with 6 hours time interval and the viability of cells was determined by taking OD_{600}nm and growth curves were constructed.

3.2.3 Cell viability assay

After treating stationary phase cells (OD_{600nm}-0.5) with different stimuli. The cells were centrifuged at 2500rpm and pellet was washed twice with sterile double distilled water and resupended in sterile double distilled water and the OD_{600nm} was adjusted to 0.1. The cells were serial diluted with sterile double distilled water (to10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). 3ul from each dilution was spotted on YPD plates and incubated at 30^{o}C for 3-4 days. The growth parameters were observed and documented.

3.2.4 FACS analysis of yeast to apoptotic markers

a) Propidium Iodide(PI) staining- Loss of Plasma membrane permeability analysis

The stressed yeast cells were pelleted at 3500 rpm at room temperature for 5 minutes and were incubated in 50mM phosphate buffer (pH 7.2) containing 1ug/ml PI for 15 minutes in dark condition. Then, cells were analyzed using FACS Caliber flow Cytometer (Becton –Dickinson) with excitation and emission wavelength settings at 480 and 530nm respectively (FL1, Detector). Data was statistically analysed by using Cellquest software (Becton –Dickinson) and PI

stained cells were observed under Carl-Zeiss Apotome microscope and documented.

b) H_2DFDA staining- Generation of reactive oxygen species analysis

The stationary phase cells were preloaded with 5ug/ml $2^1,7^o$-dichlorodihydroflourescence diacetate ((H_2DFDA), Molecular probes) and then cells were treated with various concentrations of stimuli and were analysed by FACS Caliber flow Cytometer (Becton –Dickinson) with excitation and emission wavelength settings at 304 and 551nm respectively (FL_2, Detector). Data was statistically analysed by using Cellquest software (Becton –Dickinson) and same H_2DCFDA stained cells were observed under Carl-Zeiss Apotome microscope and were documented.

c) Rhodamine123 (Rh123) staining- Loss of Mitochondrial membrane potential analysis.

The stationary phase cells were preloaded with Rh123 (10ug/ml) in YPD medium and incubated at 30^{o}C for about 30 minutes. Then the cells were washed with sterile YPD medium and subjected to various stimuli. The loss of mitochondrial membrane permeability was analysed by using FACS caliber flow Cytometer (Becton –Dickinson) with excitation and emission wavelength settings at 304 and 551nm respectively (FL_2, Detector). Data was statistically analysed by using Cellquest software (Becton –Dickinson). Rh123 stained cells were observed under Carl-Zeiss Apotome microscope and were documented.

d) DNA fragmentation assay

For analysis of nuclear fragmentation, the control and stimuli treated yeast cells were fixed in 70% ethanol at -20°C. Cells were then washed with 50mM sodium citrate and PBS (pH 7.4) and were incubated with 1 µg/ml Diaminophenylindole (DAPI) in PBS for 10 min in the dark at room temperature.

Cells were observed and photographed by using Carl-Zeiss Apoptome microscope with a UV filter.

3.2.5 Transformation of yeast with Aequorin gene and aequorin luminescence Measurements and $(Ca^{2+})_{Cyt}$ quantification.

Cells were transformed with the 2µ plasmid containing *Apo-aequorin* gene using "Improved Lithium acetate method of yeast transformation" as described by Dirick (1994). The transformed YPH-500 (Aequorin $^{+}$) was grown in SC-Ura medium to obtain OD600nm-0.5. Span down and the cells were resupended in 1ml of Sc-Ura medium. The cells were loaded with 5µl of colenterazine (50µm) for overnight. The OD_{600}nm was checked and adjusted to 10.00 and the cells were loaded once again with 5µl of colentrazine for 3hours and were analysed for cytosol calcium rise using Luminometer(TD-20/20 Turner Designs,Sunnyvale,CA). After the incubation, a cellular baseline was determined for one minute at 10seconds intervals. Then, individual stimuli were added and luminescence was recorded. Luminescence from aequorin that remained in cells at the end of an experiment was determined after treating cells with Triton-x-100 and $CaCl_2$ (2-5M). The Ca^{2+} in cytosol was calculated by using following equation.

$$[Ca^{2+}] = ((L/L_{max})^{1/3} + [118(L/L_{max})^{1/3}]—1)/ (7 \times 10^{6})-[7 \times 10^{6}(L/L_{max})^{1/3}])$$

Where L is the Luminescence intensity at any time point and L_{max} is the integrated luminescence intensity.

The detailed procedure of improved lithium acetate yeast transformation method and aequorin assay protocol is described in Appendix I.

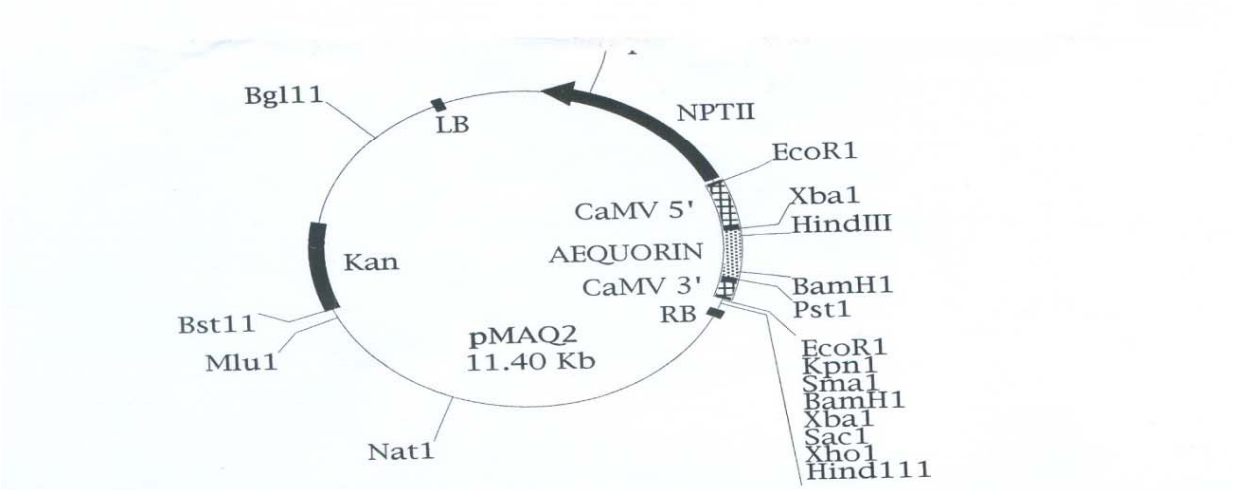


Figure 2

Plasmid pMAQ2, binary vector for transformation of higher plants with the 35S-apoaequorin gene.

Legend: CaMV 5', cauliflower mosaic virus (CaMV) 35S promoter; CaMV 3', CaMV 35S transcriptional terminator; AEQUORIN, apoaequorin coding region from cDNA clone; LB and RB, T-DNA left- and right-hand borders respectively; NPTII, neomycin transferase gene (kanamycin resistance for plants); Kan, bacterial kanamycin resistance gene. Important restriction endonuclease sites are also shown.

Fig. 9. Construct map of Apo aequorin

IV. EXPERIMENTAL RESULTS

Programmed cell death or apoptosis is a complex and well-regulated process that occurs in animal and plant systems during development, homeostasis and maintenance of integrity of multicelluar organisms and its occurrence is well reported in the eukaryotic unicellular yeast. PCD process takes place in different phases like induction, transduction and execution. The PCD process is characterized by typical morphological, biochemical and molecular features like membrane blebbing, cytosol shrinkage, DNA condensation and fragmentation, ROS (reactive oxygen species) rise and mitochondrial membrane potential (MMP) loss etc. These characteristic features differentiate PCD process from necrotopic type of cell death. These characters are scored for a number of apoptotic stimuli applied to tobacco cells and yeast cells. In our experiments with tobacco cell lines and yeast cells, we have scored for all these characters in response to each of the apoptotic stimuli to catalogue the effects of each stimulus on PCD process with respect to ROS and calcium scavengers.

In this complex process, particularly in transduction phase, second messengers and their signaling transduction pathways play an important role in execution of PCD in various organisms. Among the various secondary messengers involved in induction and execution of PCD, the role of cytosolic free calcium and reactive oxygen species (ROS) has been well established. But their onset of appearance upon induction of PCD process by a stimulus is not known.

ROS, such as super oxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals are the by-products of cellular metabolism and in plants, they are produced in relatively large amounts in mitochondria, chloroplasts, peroxisomes /glyoxisomes, plasma membrane, membranes of endoplasmic reticulum and nucleus. Environmental stresses such as bright light, cold and dehydration can increase the production of ROS in plants and animals (Foyer *et al*., 1997). ROS are reported as

the key players in PCD in both plants and animal (Jabs, 1999). ROS are thought to be utilized as signal transduction components in bringing localized PCD during plant hyper sensitive response.

ROS mediated PCD mechanism has been well defined in animal and plant systems, where as in yeast system, it is just emerging. Therefore, we conducted experiments to analyze ROS components and their role in PCD in both tobacco BY-2 protoplast and yeast cells in response to various PCD stimuli. Although ROS are widely recognized as key players in plant PCD, questions about how these molecules affect PCD remain unanswered. The cellular rise of ROS in response to various PCD stimuli is also reported in tobacco cells. We used tobacco protoplasts mainly to see cellular rise of ROS against PCD stimuli, because protoplasts are easily amenable to use in FACS and microscopic analysis as compare to intact tobacco cells.

Similarly, experiments were conducted to analyze the effect of ROS on yeast cell death in response to various PCD death stimuli, as yeast cells are also amenable to FACS analysis, owing to the ease of handling these cells. Therefore this system is handy to use than plant intact cells in studies pertaining to PCD process. To study the ROS effect on yeast system, two main ROS scavengers, TEMPOL and Glutathione were used.

Calcium is a well studied second messenger in plant signal transduction pathways. Temporary elevations of cytosolic free calcium are observed during stimuli like cold and heat shock, touch, anoxia, elicitor addition, pathogen infection, hormone administration, oxidative stress, far red light and drought. These elevations are responsible for activation or inhibition of downstream effectors such as protein kinases, gene encoding ion channels, phospholipases, oxidases, hydrolases etc. The blockage of elevation of calcium levels in cytosol may retard or inhibit above said pathways.

The temporary elevation of calcium in the cytosol due to PCD stimuli are well recorded in animal systems also. But whether this rise occurs before or after the release of ROS is not known in plant and yeast systems. Therefore, we initially tried to quantify the elevated levels of cytosolic free calcium in tobacco cells pre-incubating cells with TEMPOL. But we did not succeed due to poor loading of cells with Fluo-3 dye and also due to lower expression of chameleon construct in BY-2 cells. Therefore, yeast cells were transformed with apoaequorin gene and the transformed yeast cells with this gene were subjected to induction of PCD with various stimuli and elevated calcium levels were estimated.

Initially, tobacco BY-2 suspension cells were subjected to H_2O_2, heat, salt and mannitol stresses and morphological characters were scored and the same set of experiments were repeated by using yeast cells. Later to score ROS and MMP loss, BY-2 protoplasts and yeast cells were preloaded with H_2DCFDA and Rhodamine 123 respectively and the pre loaded cells were subjected to various treatments.

Final set of experiments were designed and conducted to understand the differences in induction of PCD process by different stimuli in BY-2 cells, protoplasts and yeast cells and the relationship between cytosolic calcium rise and ROS during PCD process against various apoptotic stimuli in yeast system and the results were confirmed using plant PCD system.

To explain the relationship between free cytosolic calcium and ROS, experiments were conducted by using calcium scavenger (EGTA) and ROS scavenger (TEMPOL) and then effect on PCD process against stimuli such as H_2O_2, heat, salt and mannitol were studied. The occurrence of cell death occurrence was documented by staining with propidium iodide by using FACS and fluorescence microscopy.

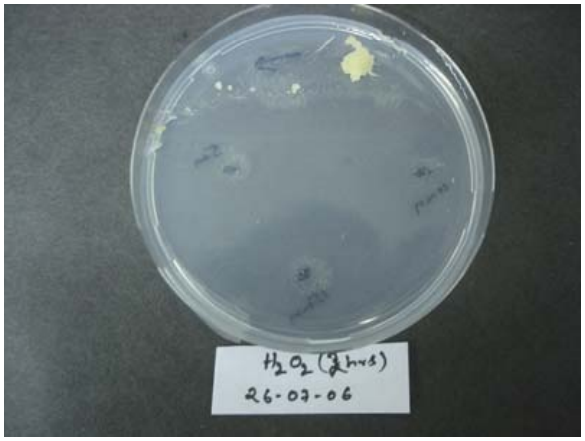
In the present investigation various experiments were conducted at the membrane Biophysics Laboratory, National Center for Biological Sciences (NCBS-TIFR Center), Bangalore and Department of Biotechnology, GKVK Campus, UAS, Bangalore and the results obtained are presented below.

4.1 Induction of PCD in tobacco Bright Yellow-2 (BY-2) cells and protoplasts using H_2O_2, Heat, NaCl and Mannitol and analysis of apoptotic characteristics.

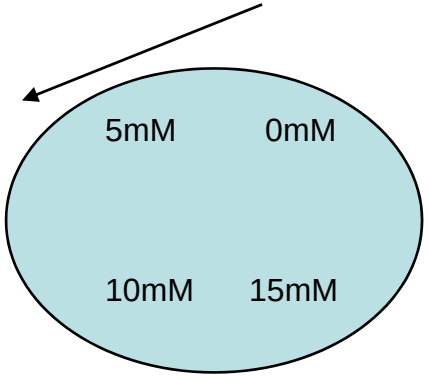
4.1.1 H_2O_2 induced programmed cell death in BY-2 cells

The ROS member, H_2O_2 was used to induce PCD in tobacco cells with 0mM, 5mM, 10mM, 15mM and 20mM concentrations initially to arrive at optimum concentration of H_2O_2 required for establishing that H_2O_2 mediated cell death is a PCD. Subsequently, only the identified optimum concentration was used in further experiments. The hydrogen peroxide treated suspension cells were spotted on MS medium. In the spot test, the H_2O_2 caused growth inhibition/retardation was observed when treated with different concentrations. The results obtained are shown in the Fig4.1.1a, As this analysis was only qualitative, cell death percentage was scored by staining the treated cells with Evans blue (only dead cells take up Evans blue) and the death percentage was calculated and interpreted.

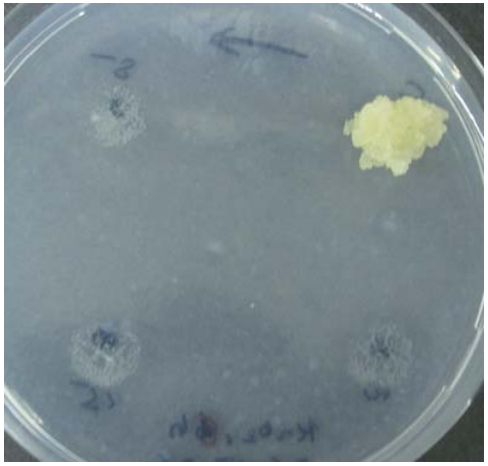
Differential percentage of cell death was observed with various concentrations of H_2O_2. Maximum death percentage (85%) was observed in 20mM H_2O_2 treated cells, while 65 % death was observed in both 15mM and 10 mM H_2O_2 treated cells, but only 35% death was recorded in 10mM H_2O_2 treated cells in the early hours (upto 16th hour) as compared to 15 mM H_2O_2 treatment. 18% percent cell death was observed in 5mM H_2O_2 treated BY-2 cells. The death pattern itself varied with concentrations and in all the treatments, the death was not observed upto 6^{th} hour treatment (Fig. 4.1.1b).



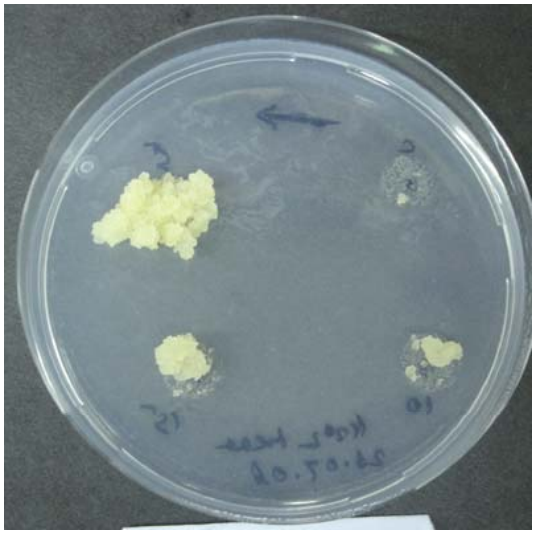
H_2O_2, 2hrs



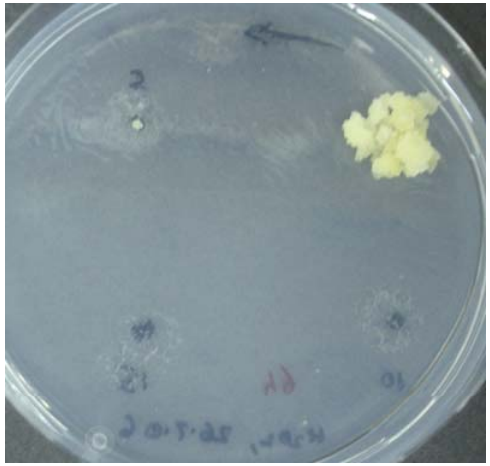
Stimulant, incubation time



H_2O_2, 4hrs



H_2O_2, 0hrs



H_2O_2, 6hrs

H_2O_2, 8hrs

Fig. 4.1.1a. Effect of H_2O_2 concentration on BY-2 cells growth

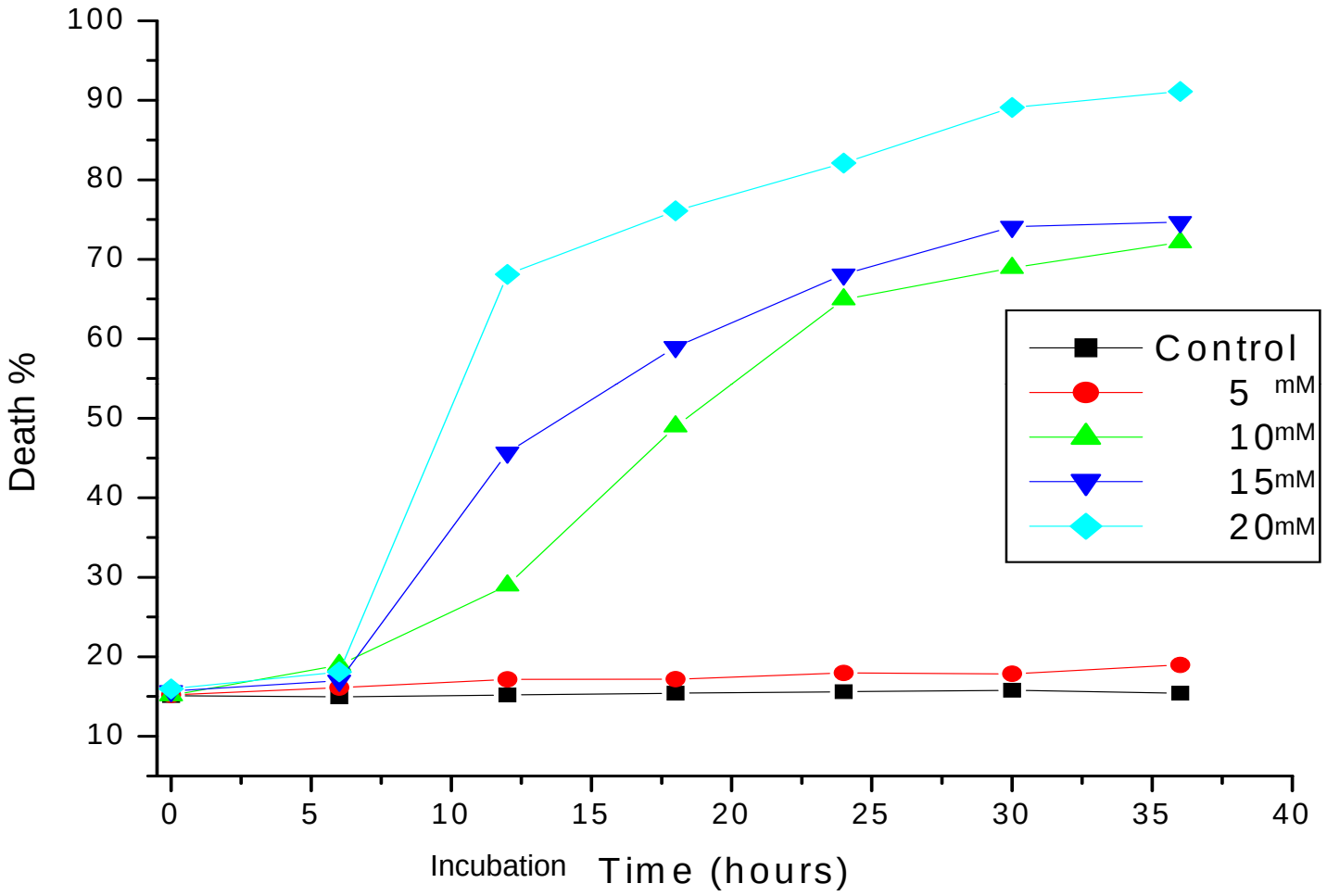


Fig.4.1.1b. Effect of H_2O_2 concentration on BY-2 cells death

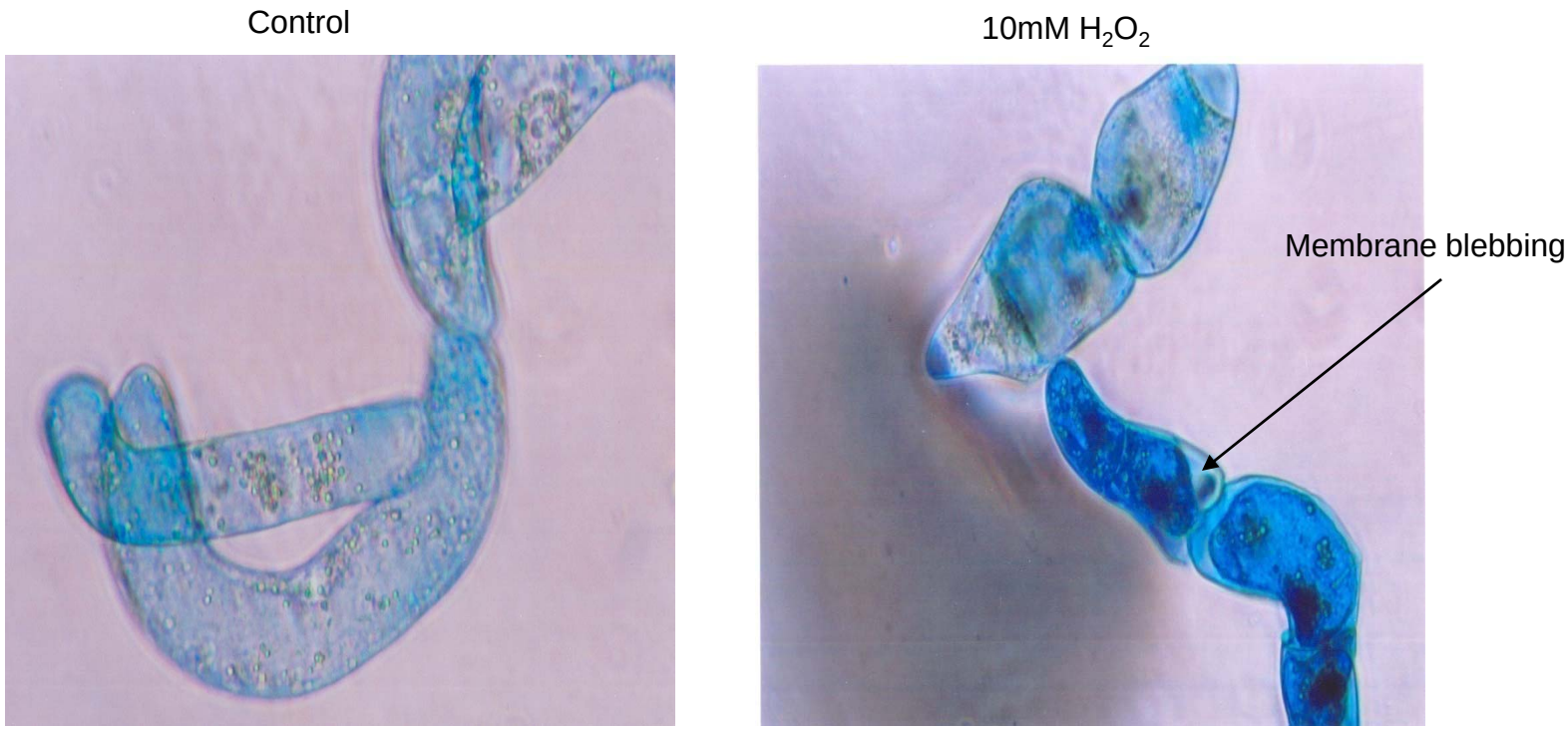


Fig 4.1.1c- BY-2 cells positive to Evans blue staining with response to H_2O_2 stress

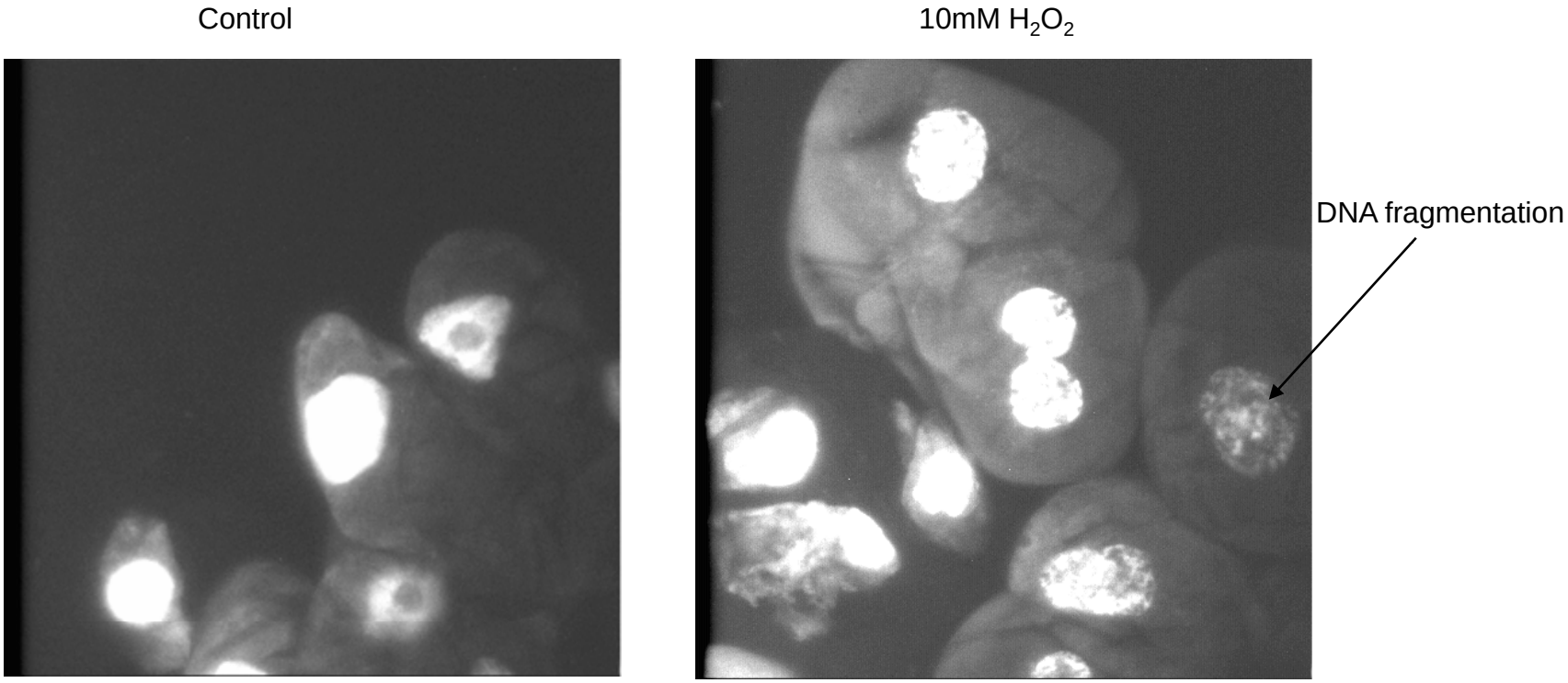


Fig. 4.1.1d. BY-2 cells showing DNA fragmentation (DAPI staining) response to H_2O_2 stress

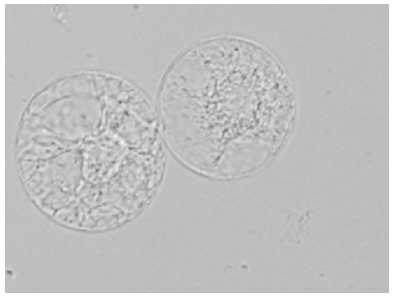
In early (before 6 hours) hours after treatment, although the cells would have lost have lost growth potential yet they did not show dead characteristics like positive reaction to Evans blue staining. In this 6 hours-time gap, PCD signal transduction pathways might occur, which is already reported in animal and plant systems. Therefore, our further experiments were conducted using 10mM H_2O_2 as a stimulus.

The morphological characteristics of 10mM H_2O_2 treated cells were examined by using microscope to verify whether the H_2O_2 mediated cell death was PCD or necrosis. It is well established that features like membrane blebbing, cytoplasm condensation, DNA condensation and fragmentation differentiate PCD type from the necrotic type of cell death.

Hydrogen peroxide treated cells showed membrane blebbing and cytosol shrinkage (Fig4.1.1c) and DAPI stained cells showed clear DNA condensation and fragmentation (Fig 4.1.1d) in treated as against control cells. These documented observed characteristic features indicted H_2O_2 induced PCD and not of necrosis.

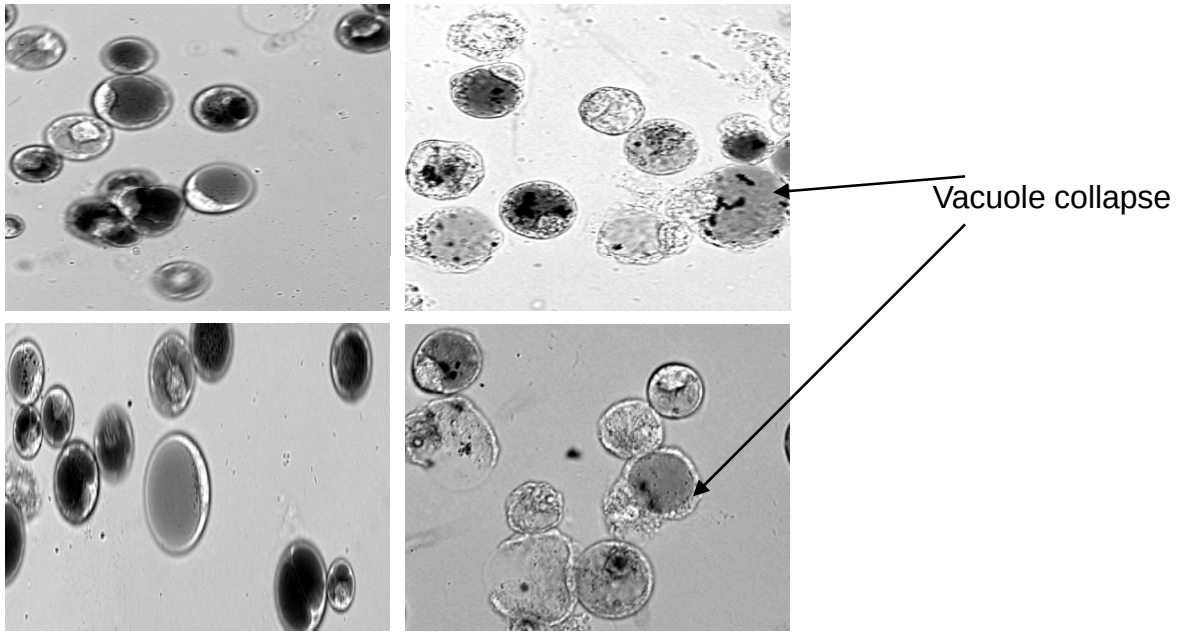
In the same manner, BY-2 protoplasts were subjected to 10mM H_2O_2 stimuli and later stained with neutral red to ascertain the vacuole nature, vacuole collapse and protoplast structure changes as observed in the treated protoplasts (Fig 4.1.1e). The Rh123 preloaded BY-2 protoplasts were also subjected to H_2O_2 stimuli to ascertain MMP collapse. In the treated protoplasts, Rh123 intensity was reduced as expected (Fig 4.1.1f).

The treated and control protoplasts were stained with PI to ascertain the cell death. Treated protoplasts showed positive reaction to PI staining compared to control (Fig 4.1.1g) and H_2DCFDA pre-loaded protoplasts showed rise in green fluorescence intensity upon adding H_2O_2. This was speculated to be due to H_2O_2 entry into the protoplasts.



C-US

Neutral red staining



Control

10mM H_2O_2

Fig. 4.1.1e. Effect H_2O_2 on BY-2 protoplasts and vacuole

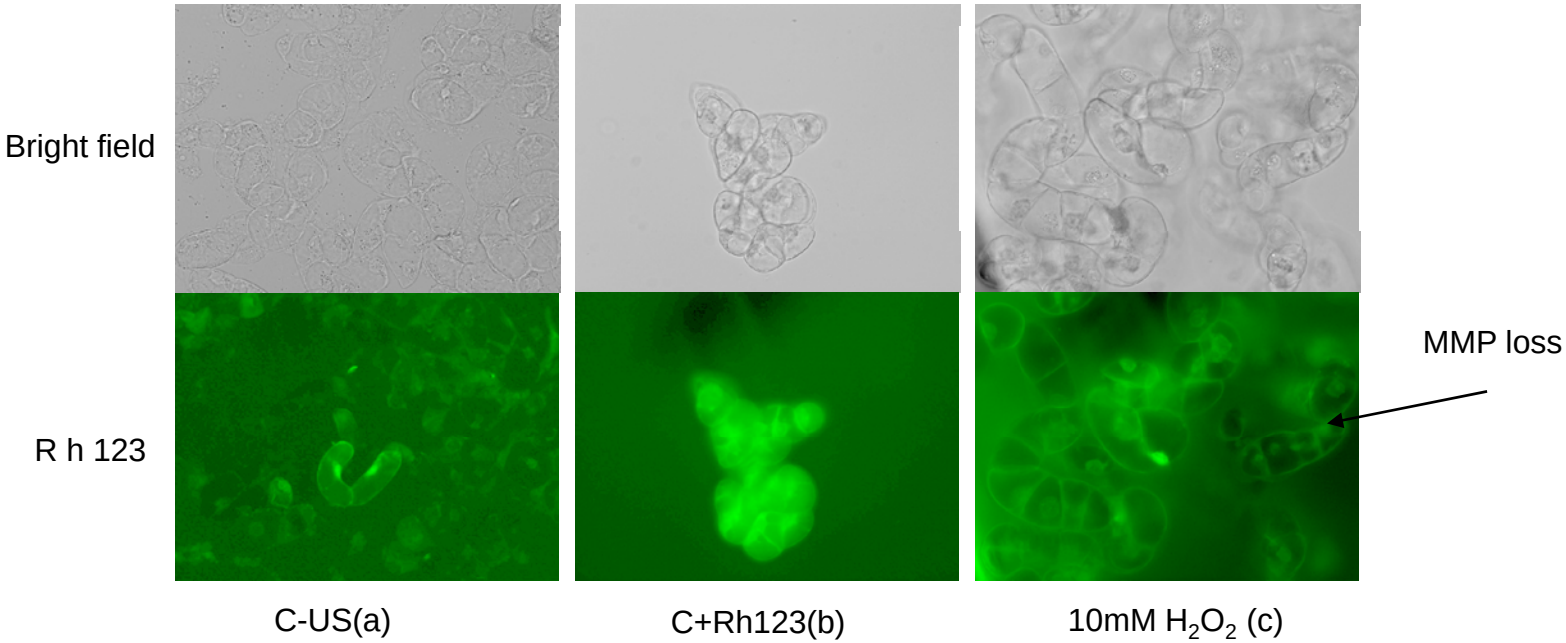


Fig 4.1.1f – Effect of H_2O_2 on BY-2 cells Mitochondrial membrane potential

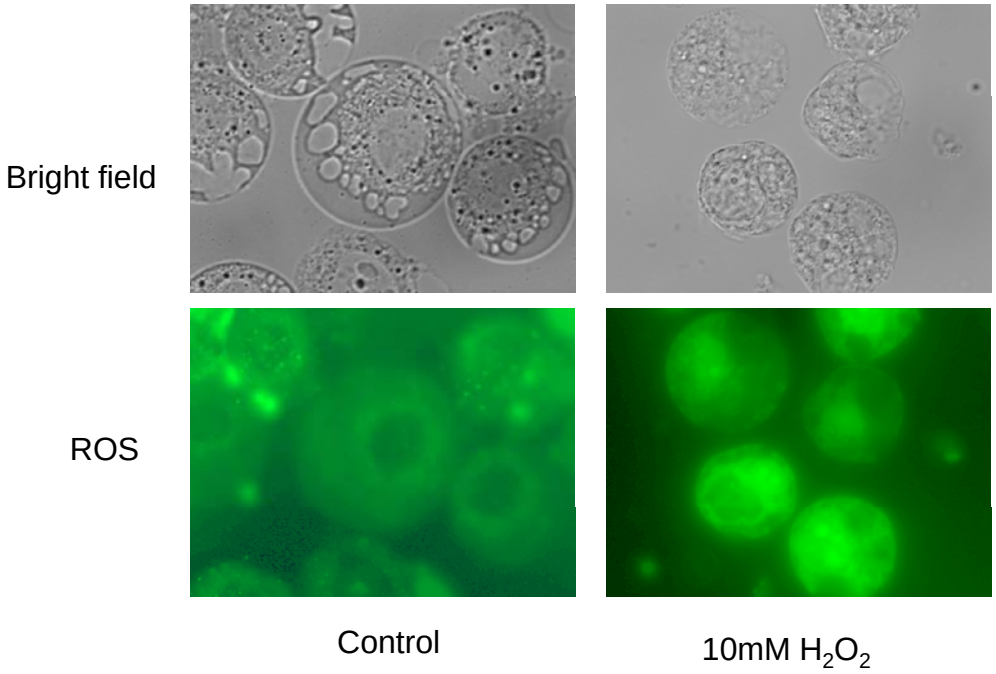


Fig. 4.1.1g. H_2O_2 treated cells showing rise in intensity of ROS

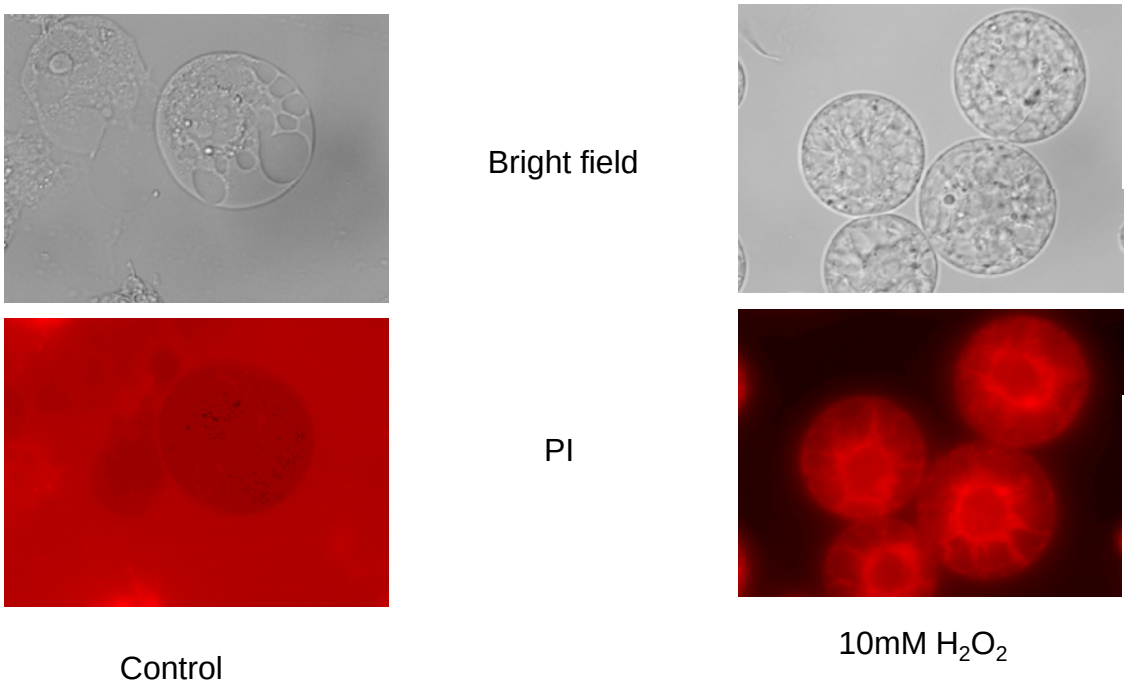


Fig. 4.1.1h H_2O_2 treated BY-2 protoplasts showing PI positive

The above said PCD morphological characteristics have been already reported in BY-2 cells against H_2O_2. We have now shown that the protoplasts also show PCD type of cell death as shown by BY-2 intact cells. As it was clearly established that H_2O_2 at 10mM concentration could induce PCD in BY-2 cells as well as in protoplasts in all the experiments that were performed later, the treatment with same concentration of H_2O_2 was used as the positive control.

4.1.2 Salinity induced programmed cell death in tobacco cells and protoplasts

Salinity is one of the major abiotic stresses experienced by the plant system during its growth and development. Salinity is a well known factor leading to yield reduction in many crop plants. Salt kills plant cells due to its toxicity and inhibits root growth and lowers photosynthetic rates. Therefore, we decided to use saline stress as a stimulus to study cell death process.

Initially BY-2 suspension cells were exposed to different NaCl concentrations of 0mM, 50mM, 100mM, 150mM, and 200mM and then spotted on MS medium to see any growth retardation or inhibition upon saline treatment. In the spot test, the BY-2 cell growth inhibition was observed in 200mM NaCl treatment within 6 hours of saline treatment. However, lower concentrations (50,100 and 150mM treated) of salt showed less or negligible growth inhibition after longer incubation periods (Fig.4.1.2a). As the results are qualitative , to assess the extent of damage by salts , cells treated with different concentrations of saline were stained with Evans blue and the death percentage was calculated (Fig.4.1.2b) at different time intervals.

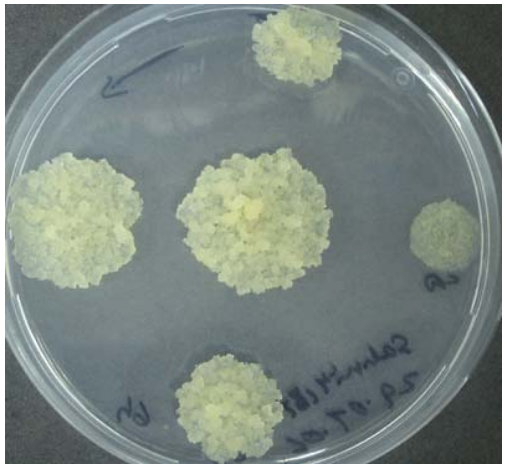
The Evans blue staining results showed the 60% cell death in 200mM NaCl treated cells as compared to control cells.40% cell death was observed in 150mM treated cells, 30-35 % cell death in 50 and 100 mM NaCl treatments. There was no substantial difference between control, 50 and 100mM treated cells with respect to

death percentage. Further, it was observed that the cells showed positive reaction to Evans blue after six hours of treatment. Therefore, further experiments were conducted by using 200mM NaCl concentration as a treatment to score for the PCD characters like membrane blebbing, cytosol shrinkage and DNA condensation and fragmentation.

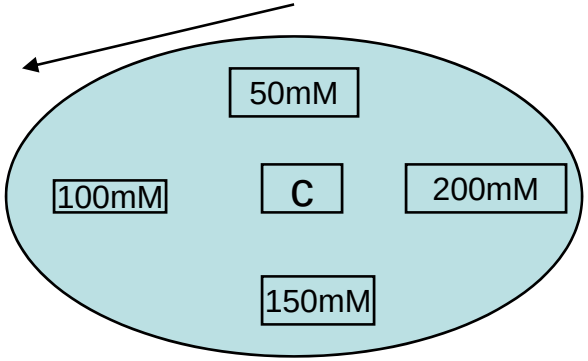
Like H_2O_2 treated cells, 200mM NaCl stressed cells were observed for PCD morphological characteristics, occurrence of Membrane blebbing, (Fig 4.1.2c), DNA condensation and fragmentation. Thus, it indicates that saline treatment induced PCD type of cell death rather than necrotic type of cell death.

Further protoplast morphology and vacuole behavior were studied against saline treatment. Though, Protoplast morphology was intact, its internal contents were condensed and fragmented. The vacuole is collapsed and its contents had been released to cytosol (Fig 4.1.2d). This character slightly differed from H_2O_2 induced protoplasts and the vacuole behavior pattern.

In PCD, initial events like MMP collapse and ROS rise are well reported in animal and plants against various apoptotic stimuli. Therefore we conducted experiments to verify the above two characters with respect to saline treatment in BY-2 protoplasts. The salinity induced cell death would probably be a PCD type rather than the necrotic type in BY-2 protoplasts. Because, Mitochondrial Membrane Potential (MMP) loss (Fig 4.1.2e) and ROS (Fig 4.1.2f) rise were observed in saline treated cells as compare to control cells. Therefore, these two characters clearly observed in both, H_2O_2 and saline treatments induced PCD cell death. Salinity stress showed other typical PCD characters like membrane blebbing, cytosol shrinkage, DNA condensation and fragmentation, MMP loss and vacuole collapse.

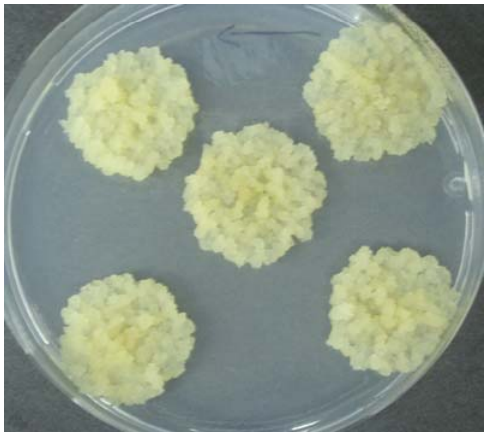


6 hrs salinity

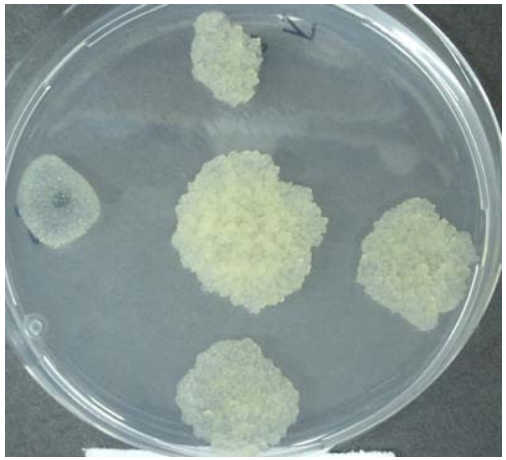


Stimulant, incubation time

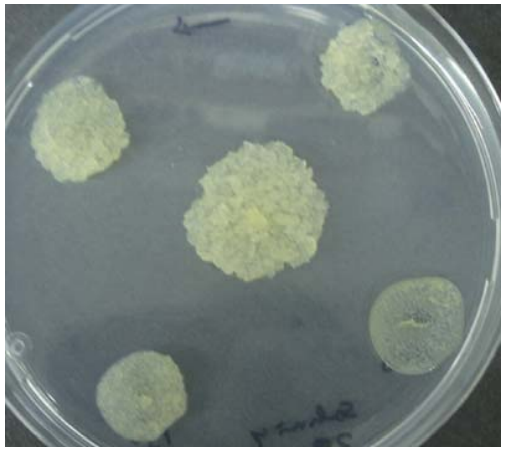
12 hrs salinity



0 hrs salinity



24 hrs salinity



48 hrs salinity

Fig. 4.1.2a. NaCl concentration and BY-2 cells growth inhibition

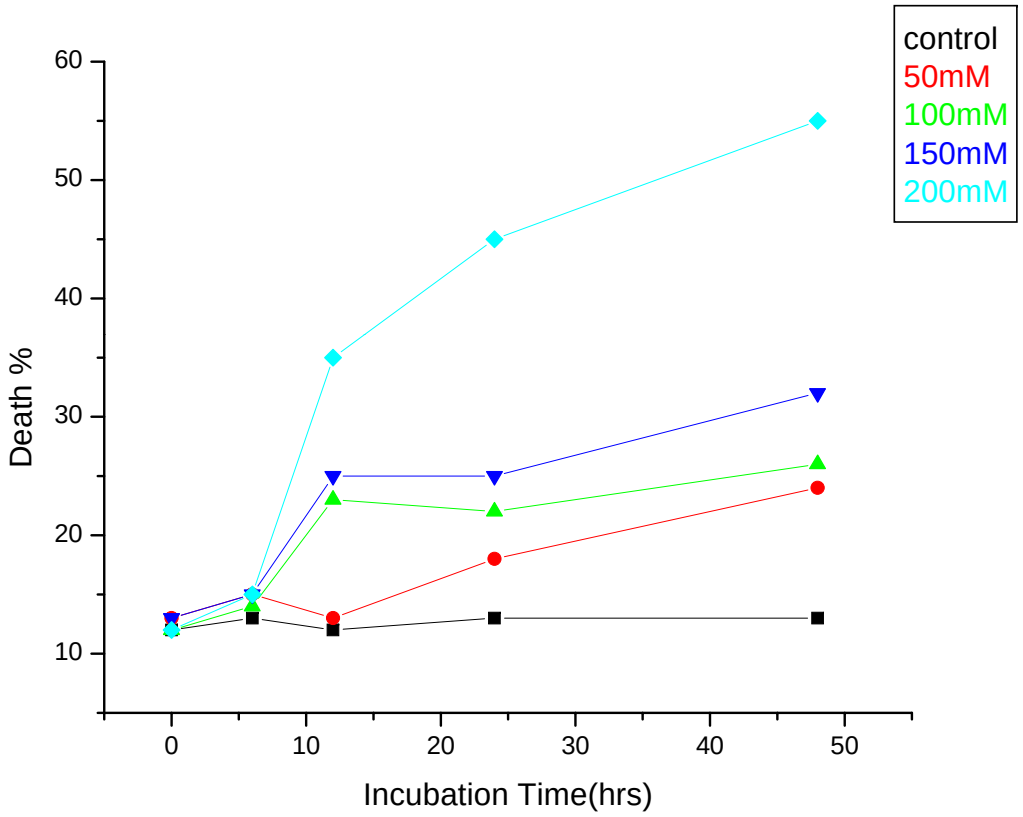


Fig. 4.1.2b. Effect of salinity on BY-2 cells death

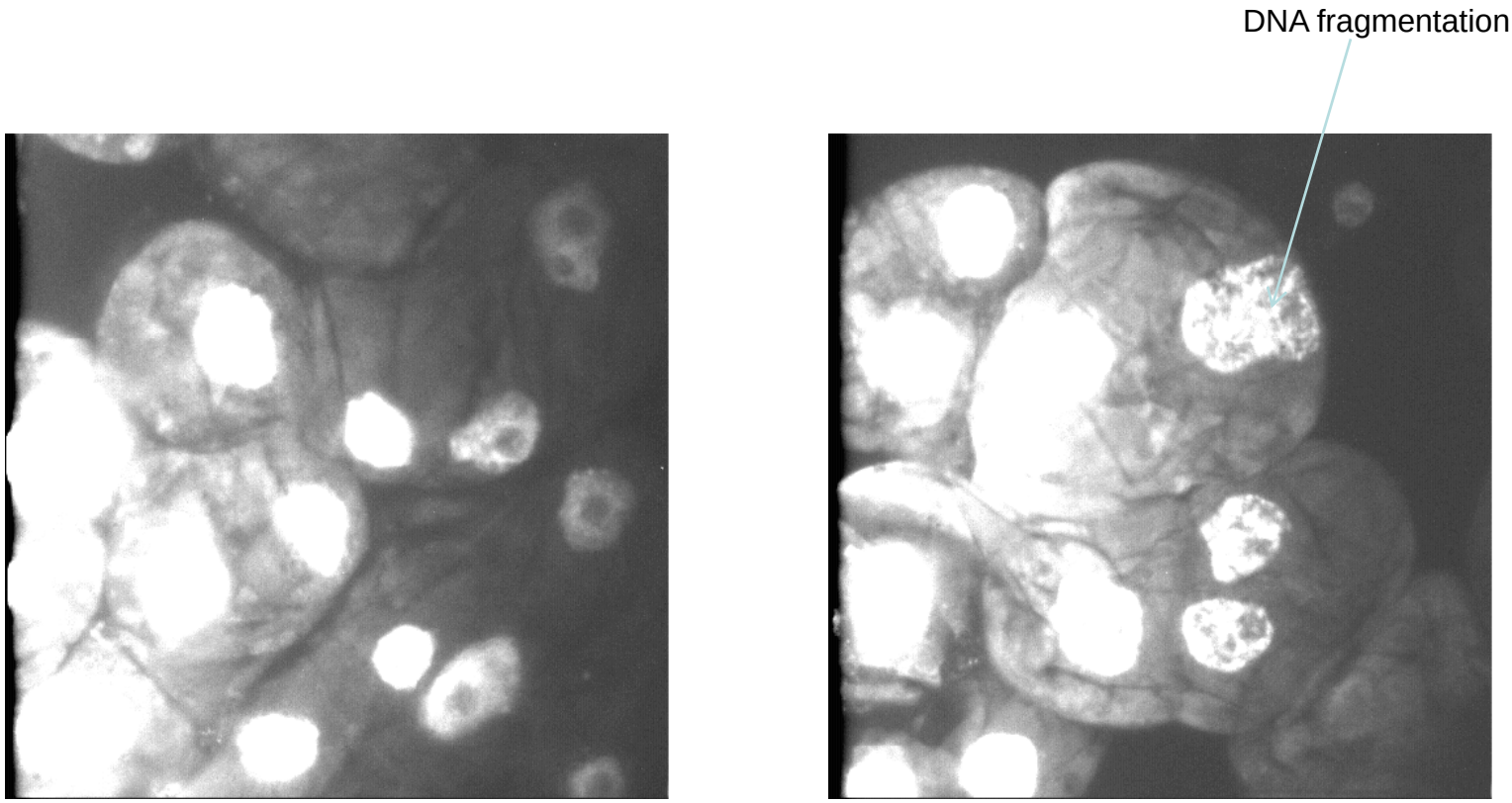
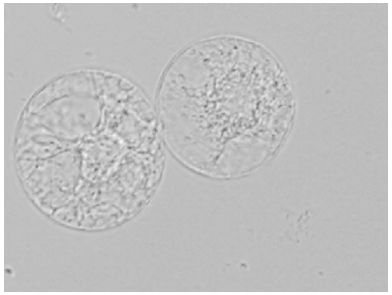


Fig. 4.1.2c. BY-2 cells showing DNA fragmentation (DAPI staining) response to Nacl stress



Control unstained

Neutral red staining

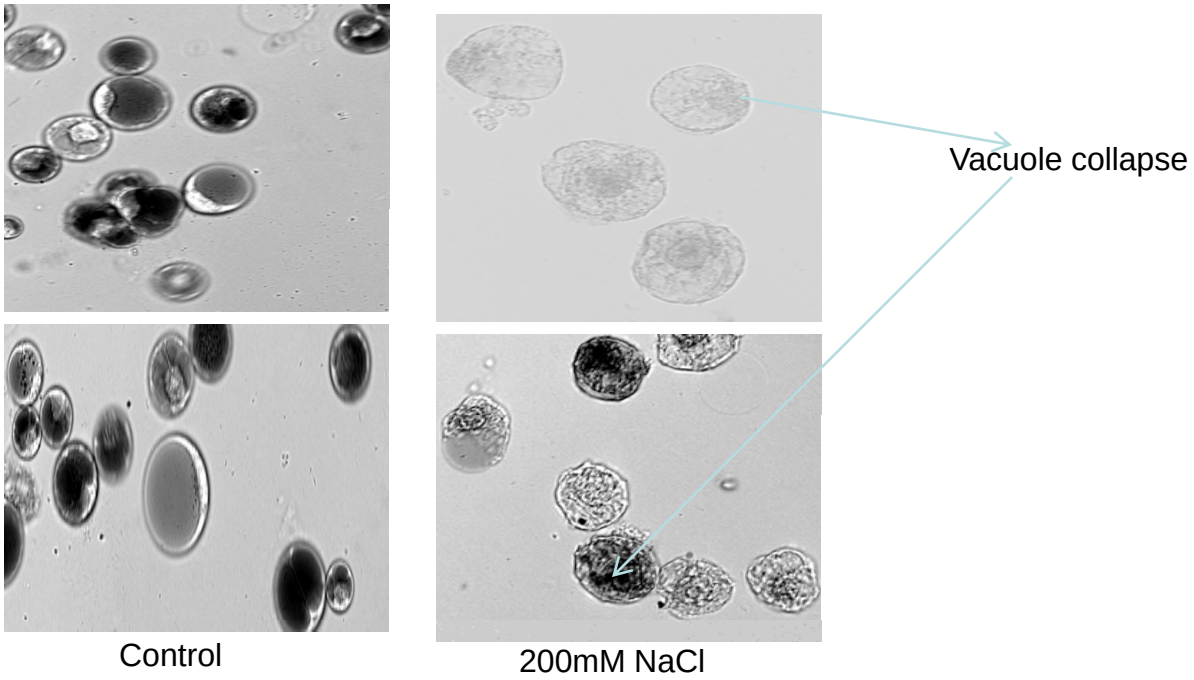


Fig 4.1.2d. Effect of NaCl on BY-2 protoplasts and vacuole

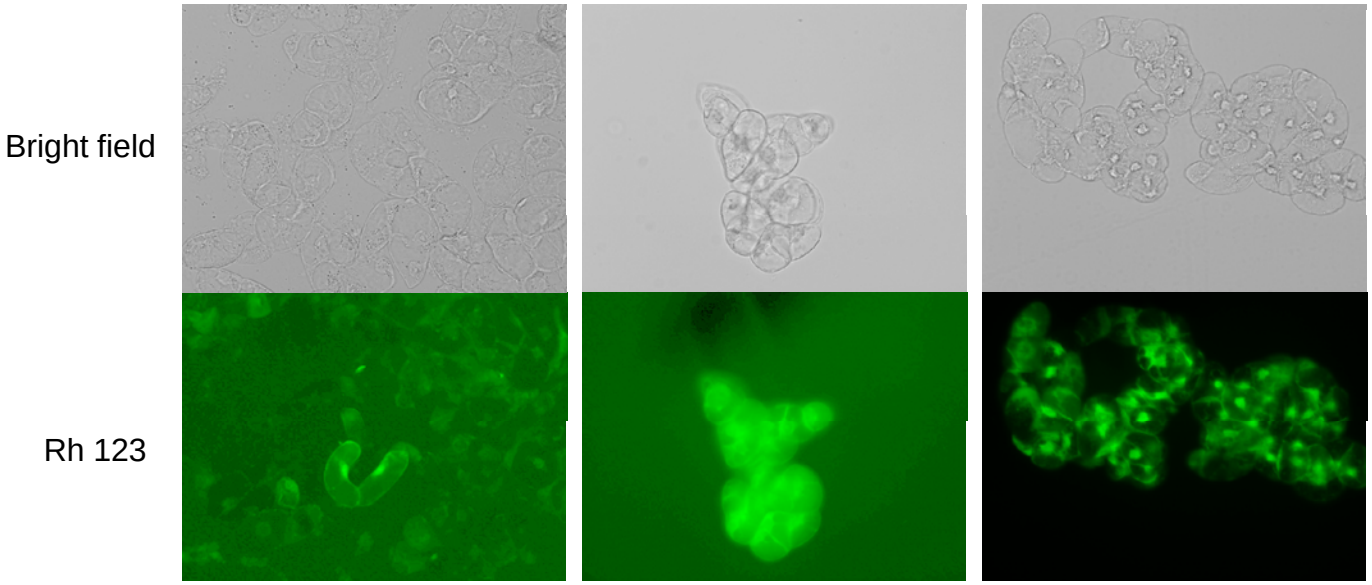


Fig 4.1.2e. Effect of NaCl treated BY-2 cells on Mitochondrial membrane potential

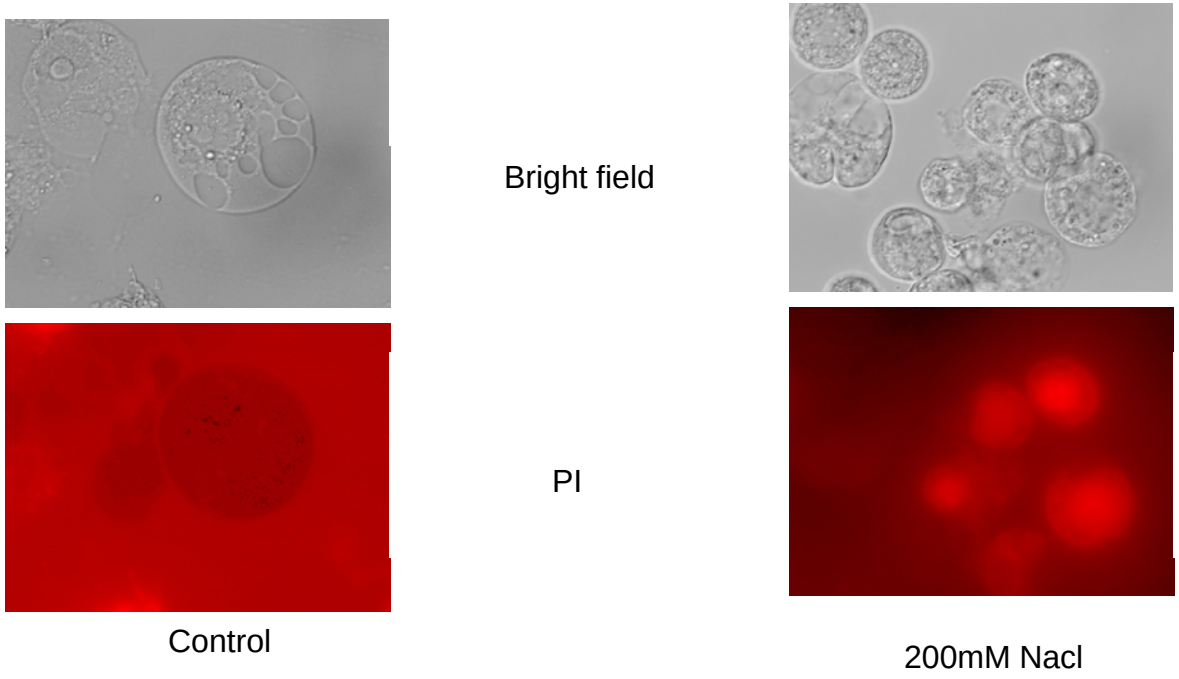


Fig. 4.1.2f. NaCl treated BY-2 protoplasts showing PI positive

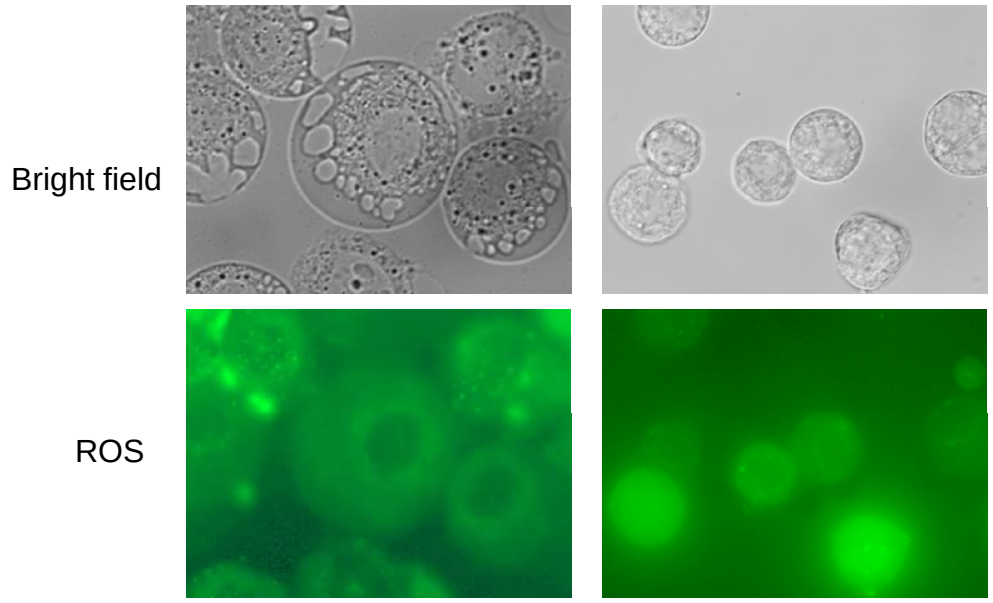


Fig. 4.1.2g. NaCl treated cells showing rise in intensity of ROS

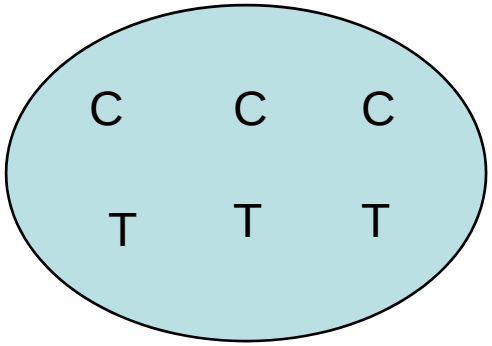
4.1.3 Heat induced Programmed cell death in tobacco protoplasts and cells

Like Salinity, heat is one of the abiotic stress that affects plant growth and development. High temperatures cause an array of morpho-anatomical, physiological and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in the economic yield. Furthermore, throughout plant ontogeny, enhanced expression of a variety of heat shock proteins, other stress-related proteins, and production of reactive oxygen species (ROS) constitute major plant responses to heat stress. In order to cope with heat stress, plants implement various mechanisms, including maintenance of membrane stability, scavenging of ROS, production of antioxidants, accumulation and adjustment of compatible solutes, induction of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades.

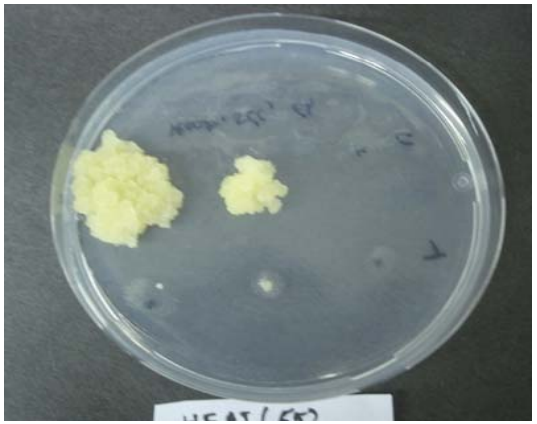
Heat, has already been shown as an apopotic stimulus in the animal system. But in plant cells this has been demonstrated, but few characters like ROS rise, vacuole collapse are not studied and therefore heat stress was selected as one of the PCD stimuli to induce cell death in BY-2 cells and protoplasts. BY-2 cells are exposed to 45^{o}C, 55^{o}C and 65^{o}C temperatures for about 10 minutes and stained with Evans blue and cell death percentage was calculated. 55% of cell death was observed at 55^{o}C and 80% of cell death observed at 65^{o}C (Fig 4.1.3a) and no cell death was observed when cells were exposed to 45^{o}C. The spot test was carried out on 55^{o}C treatment. The growth inhibition was observed after 6 hrs after treatment (Fig.4.1.3b) and to do further experiments 55^{o}C heat shock was used and PCD characters were scored.

Heat (55^{o}C) treated BY-2 cells and protoplasts were examined for typical PCD characteristics. BY-2 cells showed clear membrane blebbing, cytosol shrinkage, DNA condensation and fragmentation like in H_2O_2 stressed cells (Fig 4.1.3c), ROS rise (Fig 4.1.3g) and MMP collapse (Fig 4.1.3e) were observed

Heat, 55°C, 6hrs



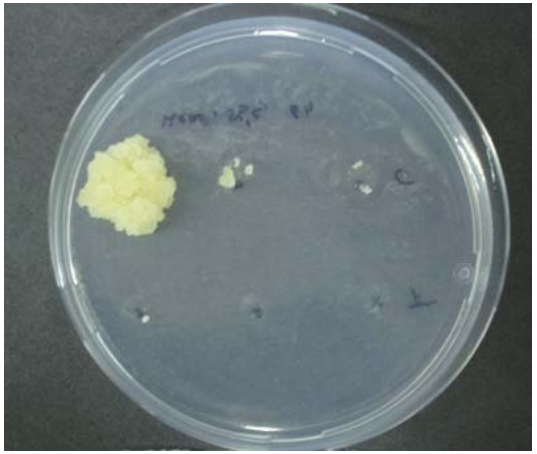
Heat, incubation time



Heat, 55°c,12hrs

Heat, 55°C, 18hrs

Heat, 55°c, 0hrs



Heat, 55°C,24hrs

Fig. 4.1.3a. Effect of Heat stress on BY-2 cells growth

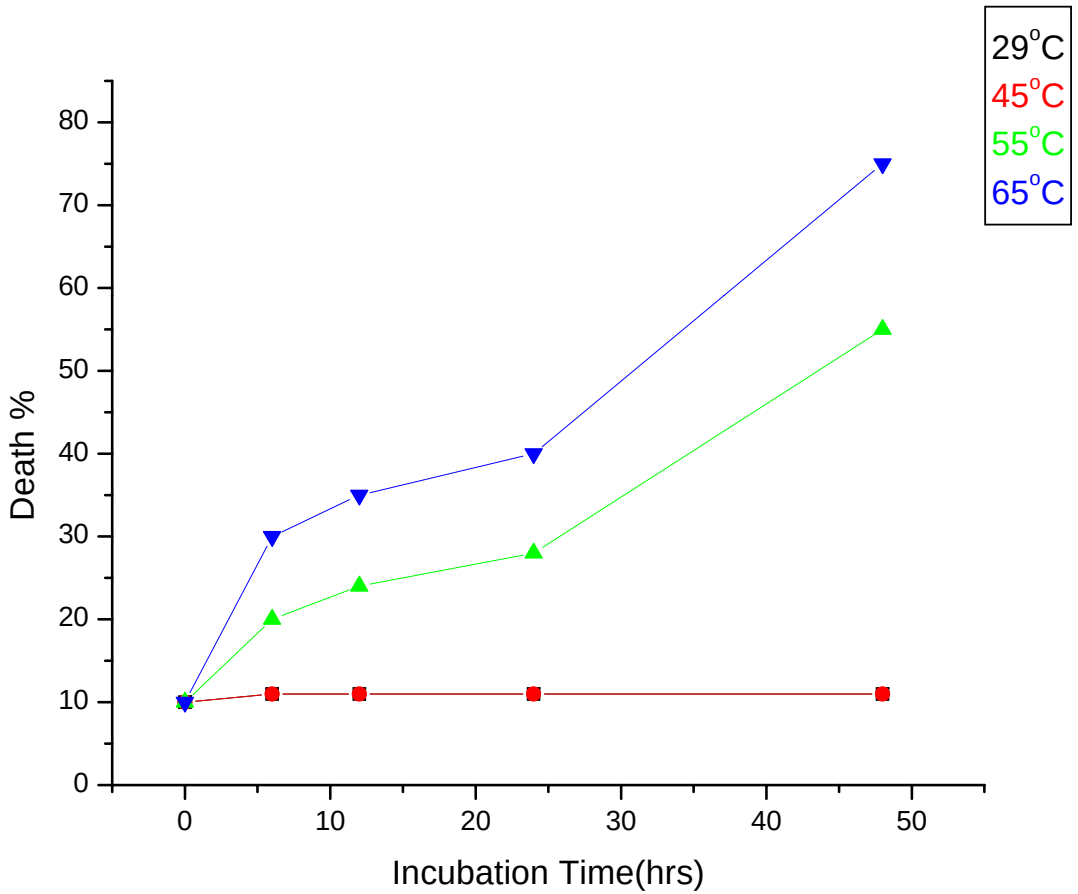


Fig. 4.1.3b. Effect of heat on BY-2 cells death

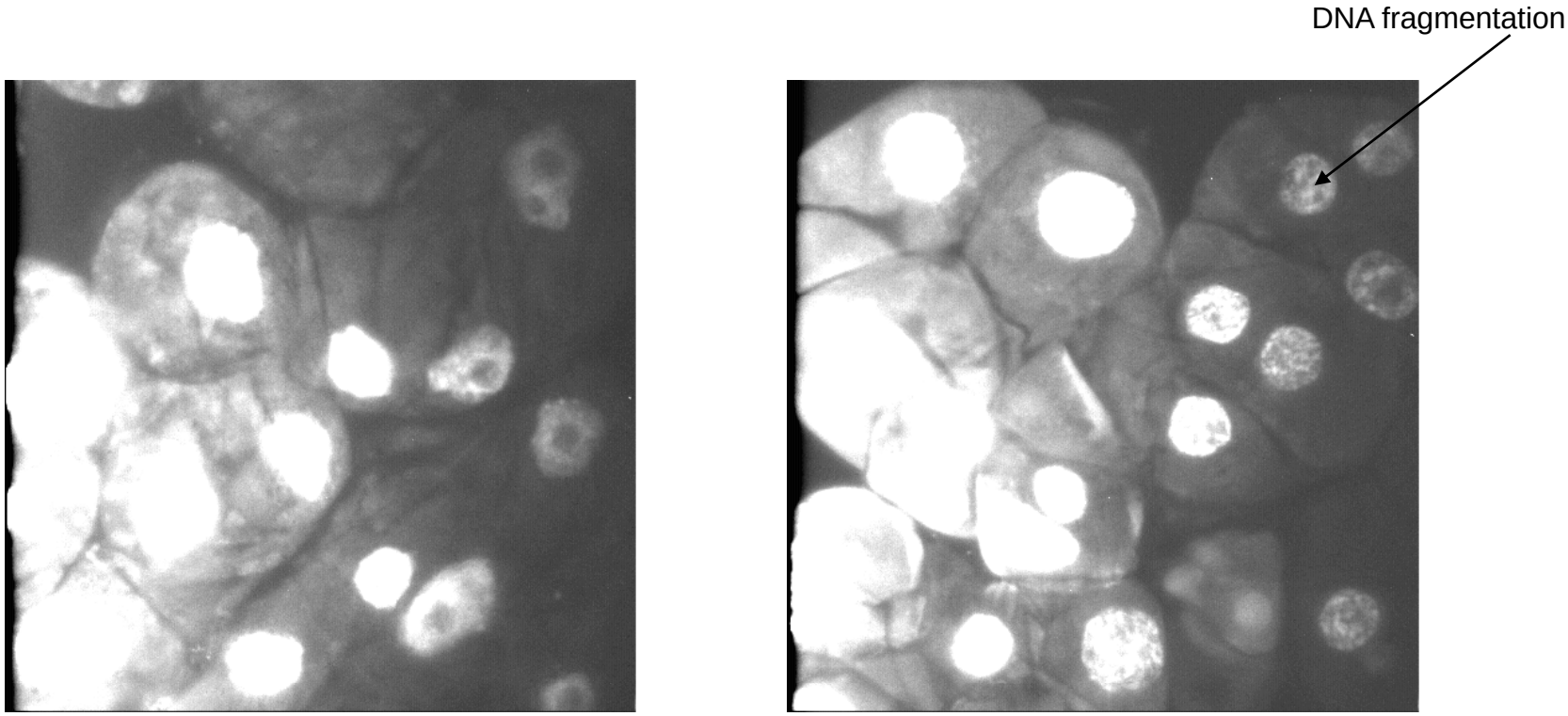
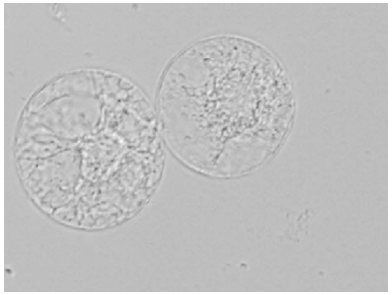
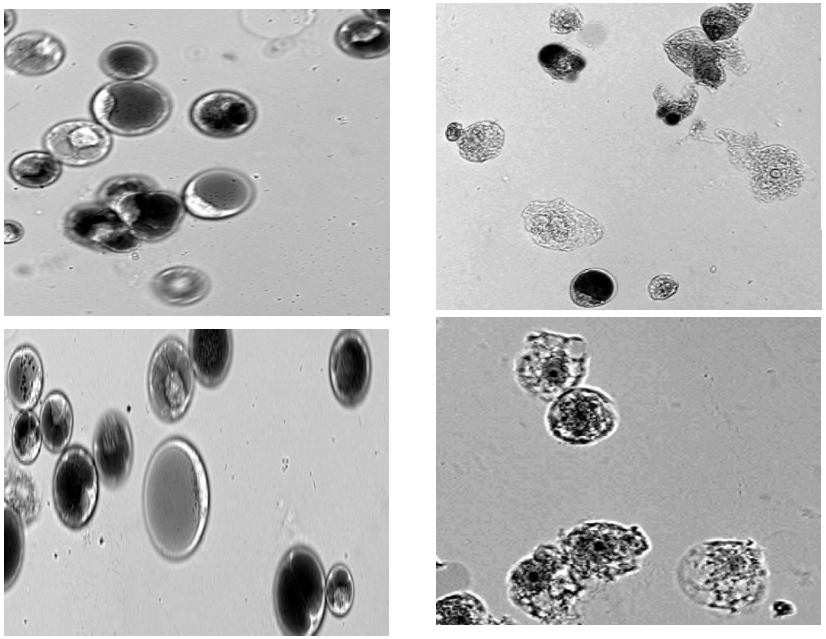


Fig. 4.1.3c. BY-2 cells showing DNA fragmentation (DAPI staining) response to Heat stress



C-US

Neutral red staining



Control

55^{o}C

Fig. 4.1.3d. Effect of Heat stress on BY-2 protoplasts and vacuole

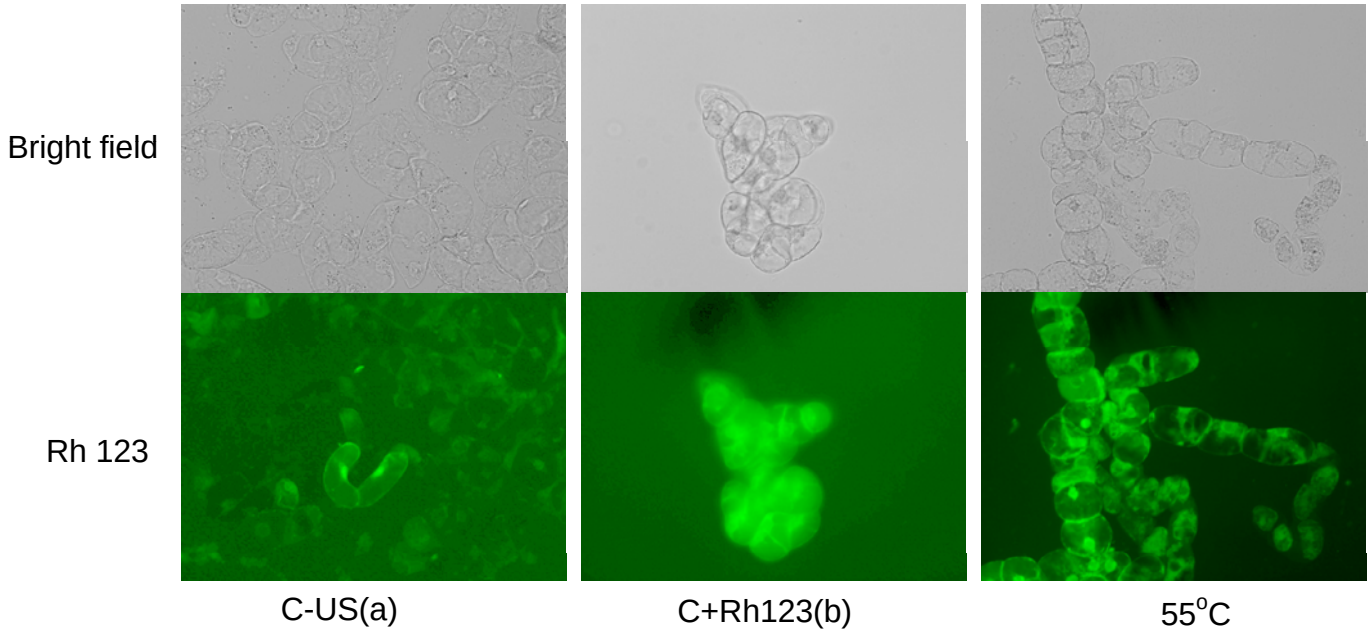


Fig. 4.1.3e. Heat treated BY-2 cells showing collapse of Mitochondrial membrane potential

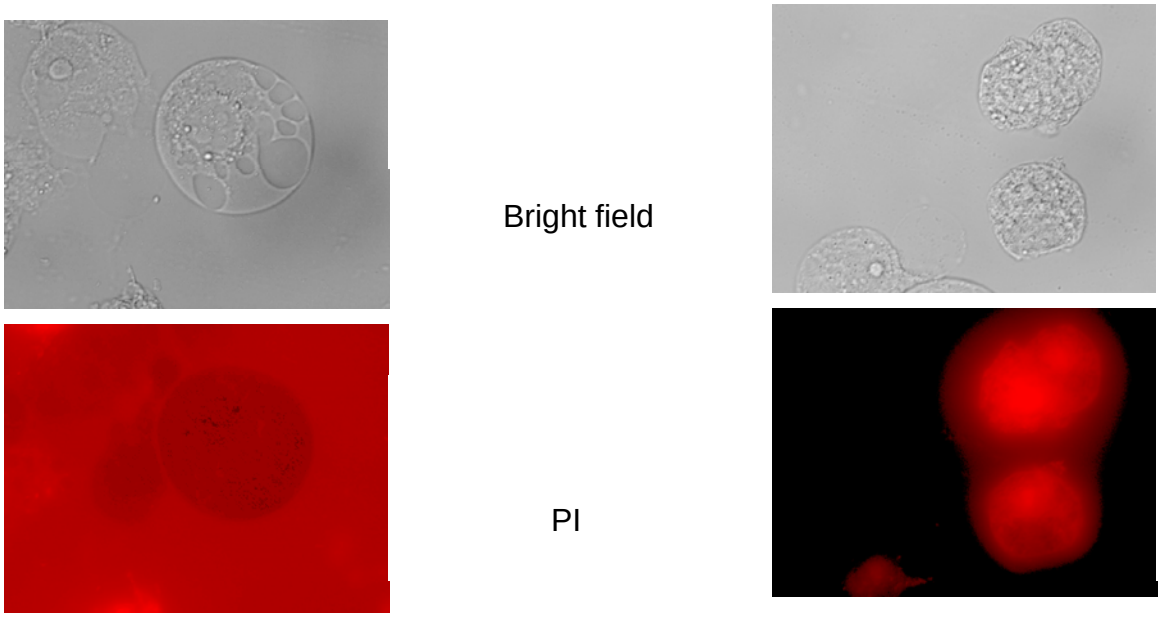


Fig. 4.1.3f. Heat treated BY-2 protoplasts showing PI positive

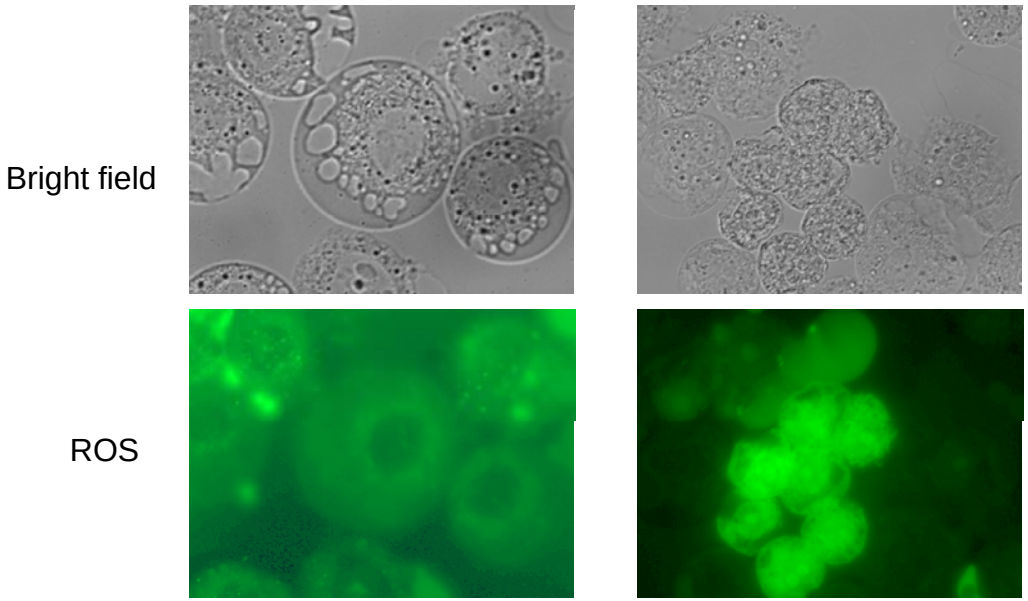


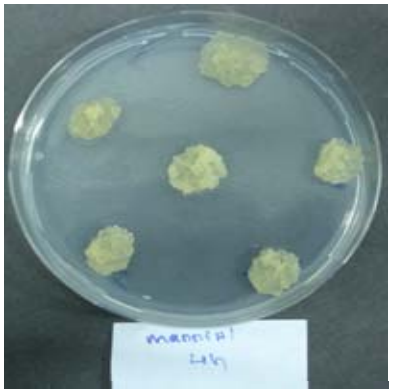
Fig. 4.1.3g. Heat treated protoplasts showing rise in intensity of ROS

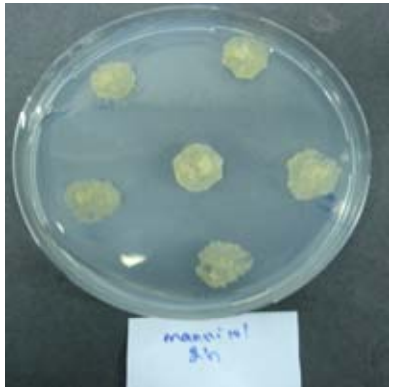
during heat stress and the protoplasts showed positive reaction to PI (Fig 4.1.3d). These observations were similar to the observations made with H_2O_2 and saline treatments.

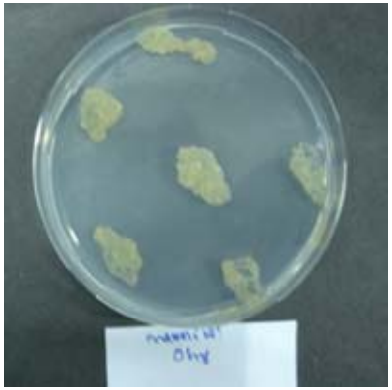
Further, protoplasts morphology and vacuolar characteristics were carefully observed and compared with H_2O_2 and saline treatments. Protoplast maintained its shape. However the contents were condensed and vacuole collapse was evident and its content might have been released into cytosol. There was slight difference in protoplast morphology when compared to H_2O_2 and saline treated protoplasts. Even though H_2O_2, salinity and heat stresses were shown to cause programmed cell death, protoplast morphology and vacuole collapse varied with respect to H_2O_2, salinity and heat stress. In H_2O_2 treatment, protoplasts normally maintained their shape, where as salinity and heat-stress showed changes in the shape. With the initial experiments, it was established that the three (H_2O_2, salinity and heat) studied apoptotic stimuli induced cell death in BY-2 cells and protoplasts that was more like PCD rather that necrotic type of cell death .

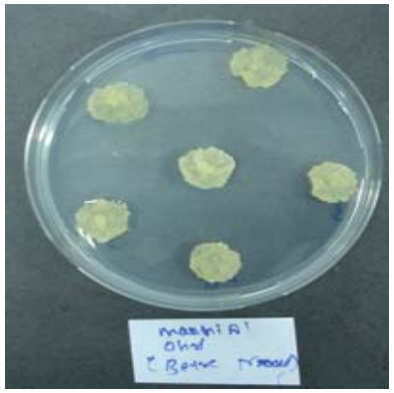
4.1.4 Mannitol does not induce any cell death in BY-2 cells and protoplasts

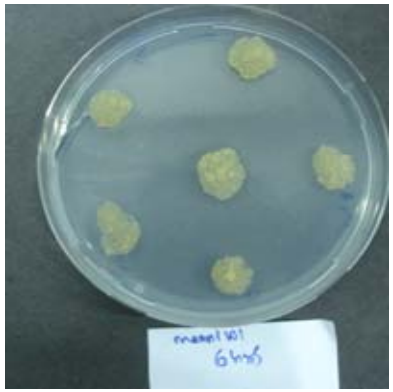
Mannitol, which is not universally produced by plants, has been reported to mitigate abiotic stress in several dicot species. Mannitol is one of the mostly used osmoticum in protoplast preparation at lower concentration. Therefore it has not been reported as a PCD stimulus and its accumulation is well reported during water and saline stress conditions. To test whether mannitol induces PCD in cell lines, we tested five concentrations of mannitol. The five concentrations include (1, 2, 3, 4 and 5 %) used in the experiments. The treated BY-2 cells and protoplasts of tobacco cells did not show any symptoms of growth inhibition (Fig 4.1.4a) as well as the positive reaction to Evans blue staining. Treated BY-2 cells did not show any ROS rise, MMP loss (Fig 4.1.4c) and DNA condensation and fragmentation. Mannitol treated protoplasts did not show any defects in vacuole











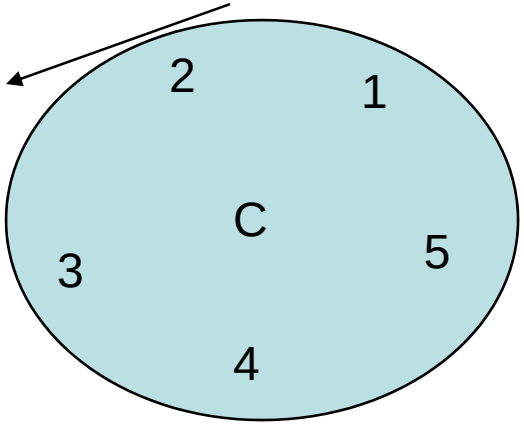
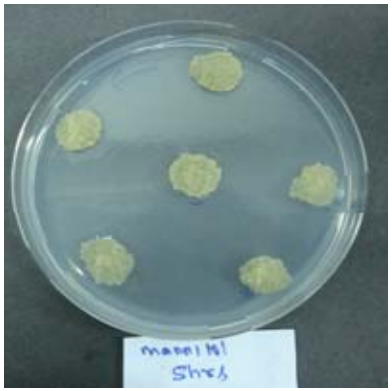
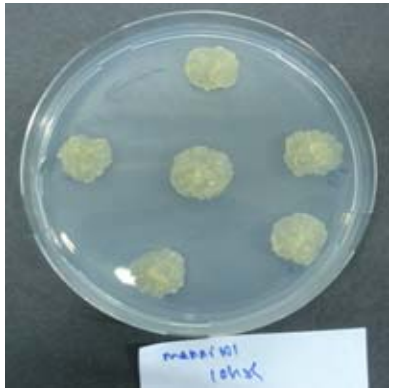
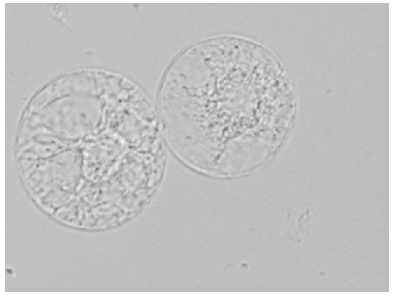


Fig. 4.1.4a. Effect of Mannitol on BY-2 cells growth







C-US

Neutral red staining

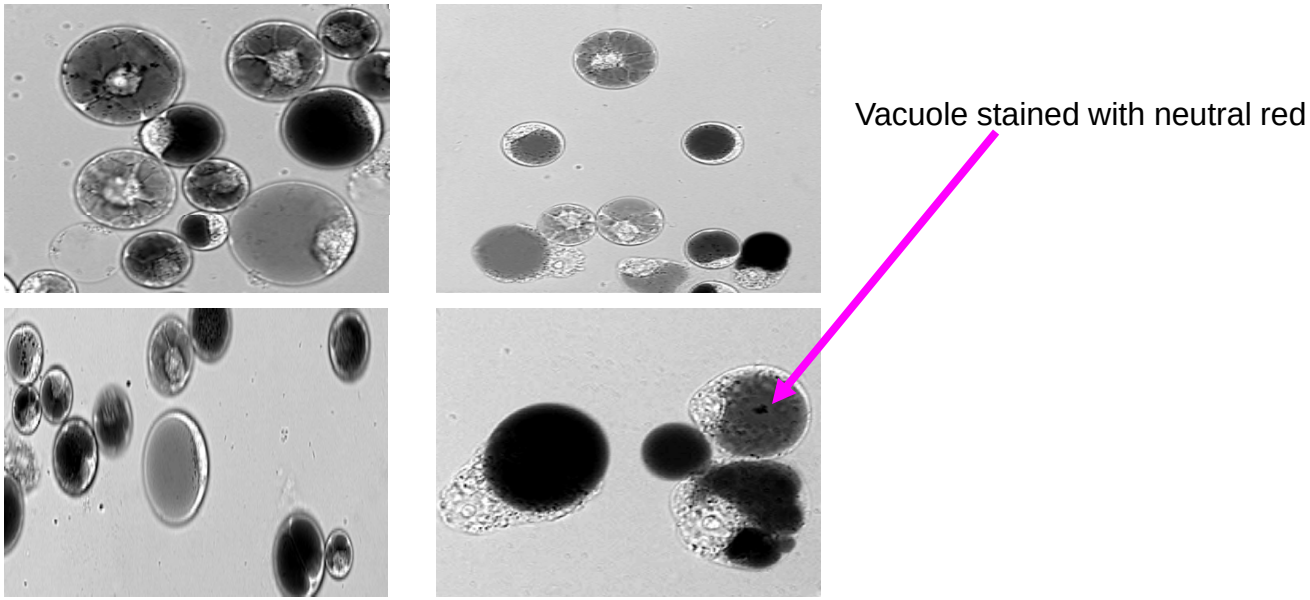


Fig. 4.1.4b. Effect of Mannitol on BY-2 protoplasts and vacuole

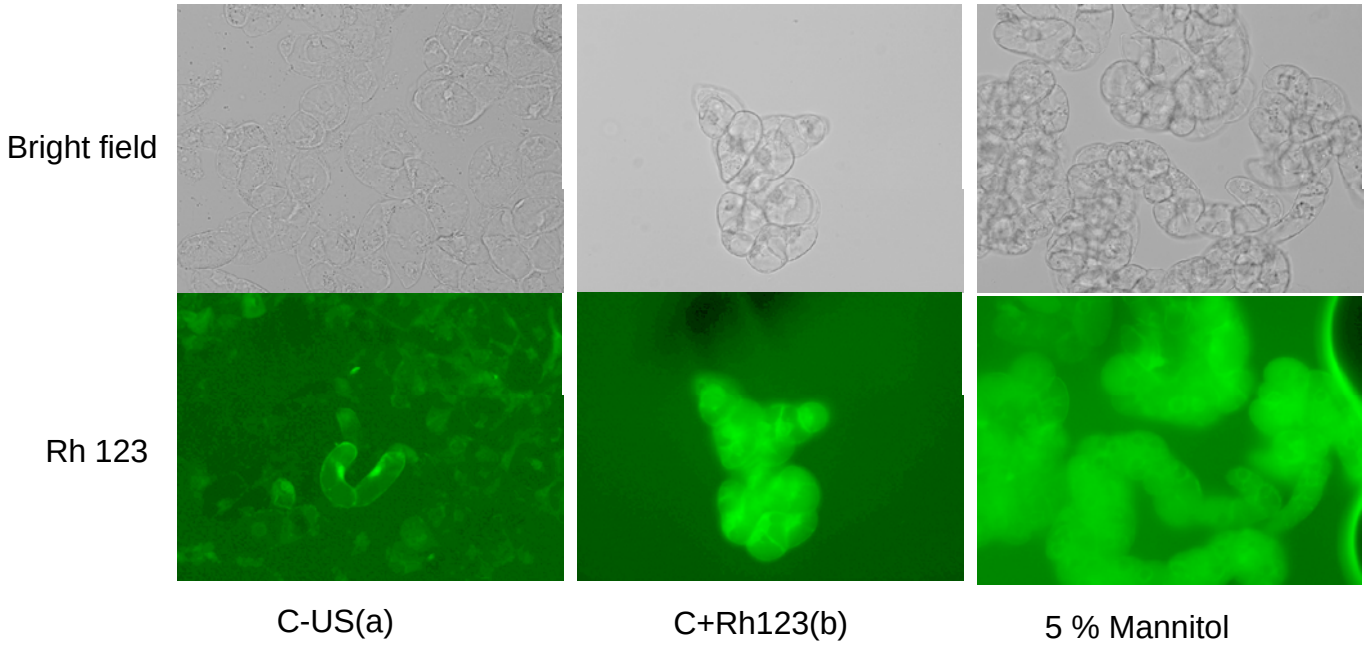


Fig. 4.1.4c. Effect of mannitol on BY-2 cells on Mitochondrial membrane potential

collapse (Fig 4.1.4b). Therefore upto 5% mannitol will not induce any type of cell death in BY-2 cells and it is not a stimulus to cell death.

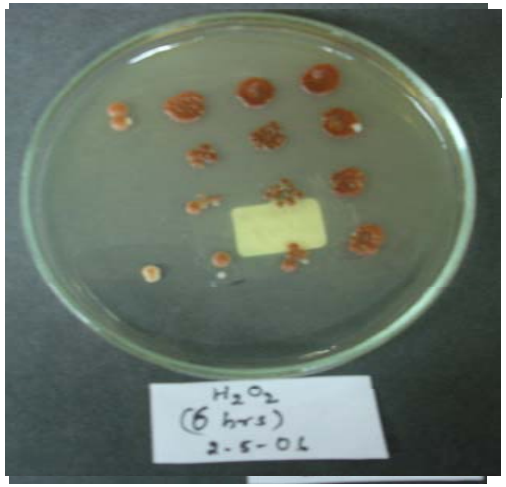
4.2 Induction of apoptosis in yeast (YPH 500) by using H_2O_2, heat, NaCl and mannitol and analysis of apoptotic characteristics.

Studies on the unicellular, eukaryotic yeast (*Saccharomyces cerevisiae)* are promising because it is a model system with well studied physiology, morphology, biochemistry, genetics and molecular biology. It is easily amenable to molecular studies and easy to use in FACS and microscopic experiments. Few reports have suggested that apoptosis –like programs are present in *S.cerevisie.* This information has been obtained by exogenously expressing pro or anti apoptotic mammalian genes in the yeast system and scoring for apoptotic characters.

Later, yeast was used as a model organism to study genetic and cytological aspects of apoptotic process reported against known plant and animal apoptotic stimuli ranging from H_2O_2 to viral toxins. Therefore, experiments were conducted in yeast strain YPH-500 by using animal and plant apoptotic stimuli like H_2O_2, heat, saline as apoptotic stimuli to induce cell death and score apoptotic characters to establish relationship between ROS generation and calcium.

4.2.1 H_2O_2 induces programmed cell death in yeast

Wild type yeast was exposed to H_2O_2, which is known to induce apoptosis in budding yeast. Yeast cells in the exponential phase of growth were used to test apoptosis process by using the lower concentrations (1mM, 2mM, 3mM, 4mM and 5mM) and higher concentrations (10mM and 15mM) of H_2O_2. At every 2 hours intervals, optical density at 600nm was recorded to check the cell division arrest. The growth inhibition was clearly observed even at 1mM H_2O_2 and more growth arrest was observed at higher concentrations of H_2O_2. Later to explain its apoptotic



concentration

15mM	10mM	5mM	C	
				10^{-1}
				10^{-2}
				10^{-3}
				10^{-4}

dilution

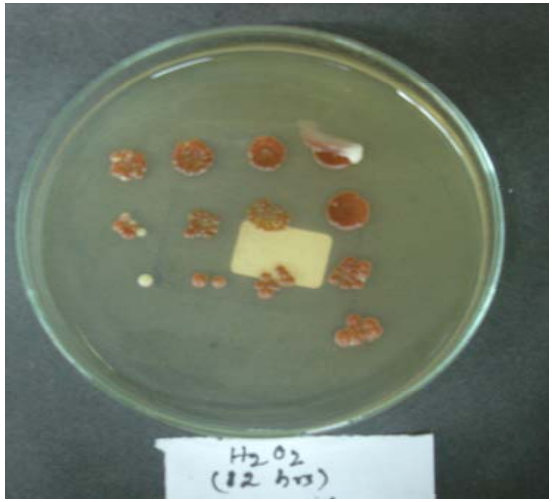


Fig. 4.2.1a. Effect of H_2O_2 on yeast cells growth

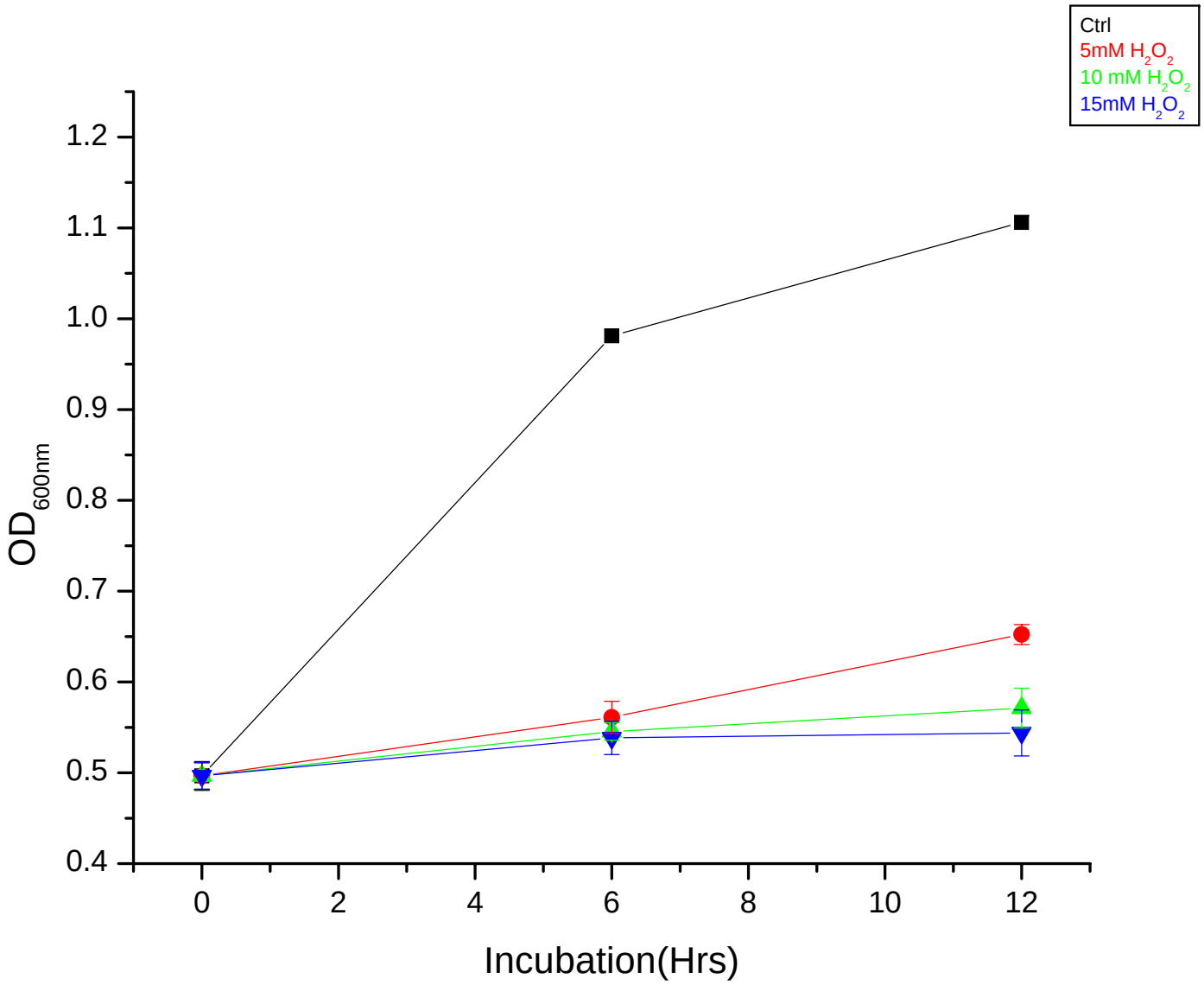


Fig. 4.2.1b. The effect of H_2O_2 on yeast cells growth response curve

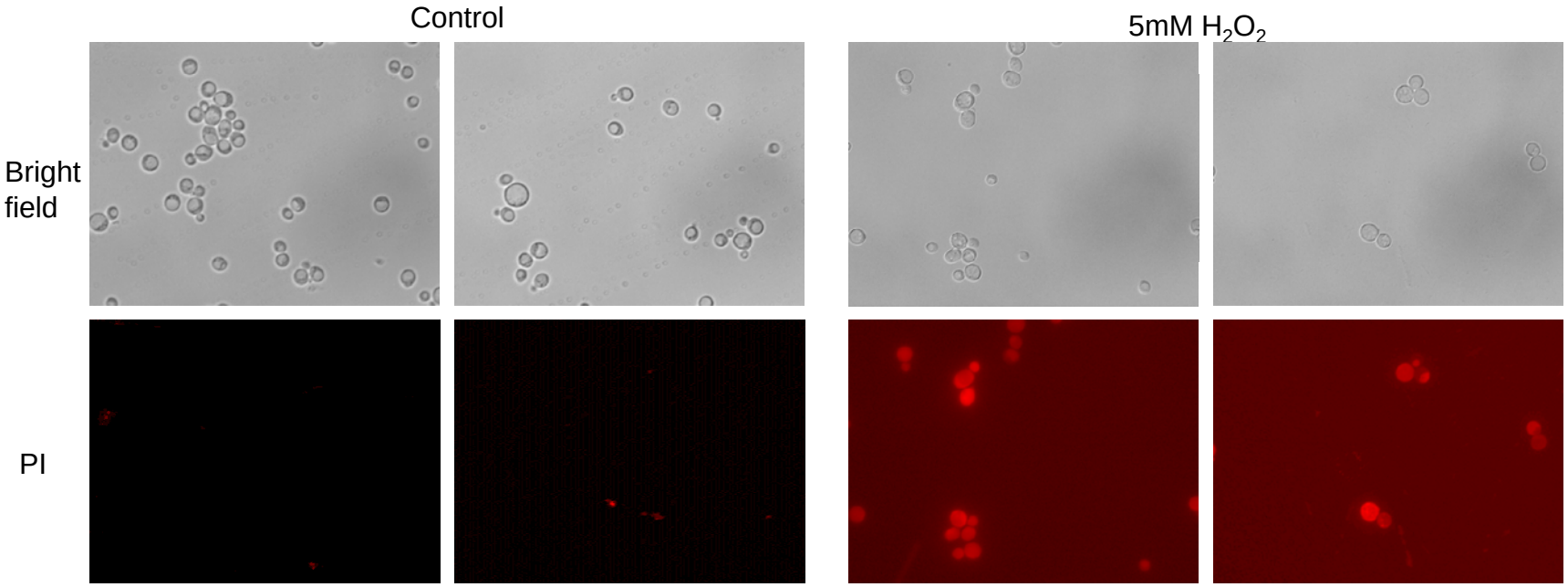


Fig 4.2.1c-a

Fig 4.2.1d-b

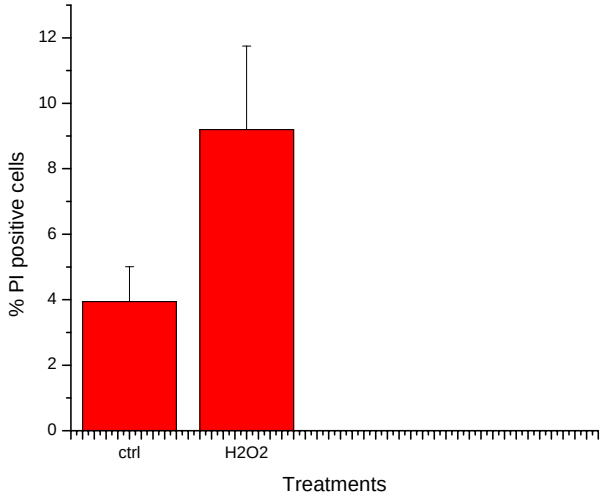


Fig 4.2.1c-c

Hydrogen peroxide treated cells showing PI positive

a) Control
b) 5mM H_2O_2 treated cells
c) FACS data

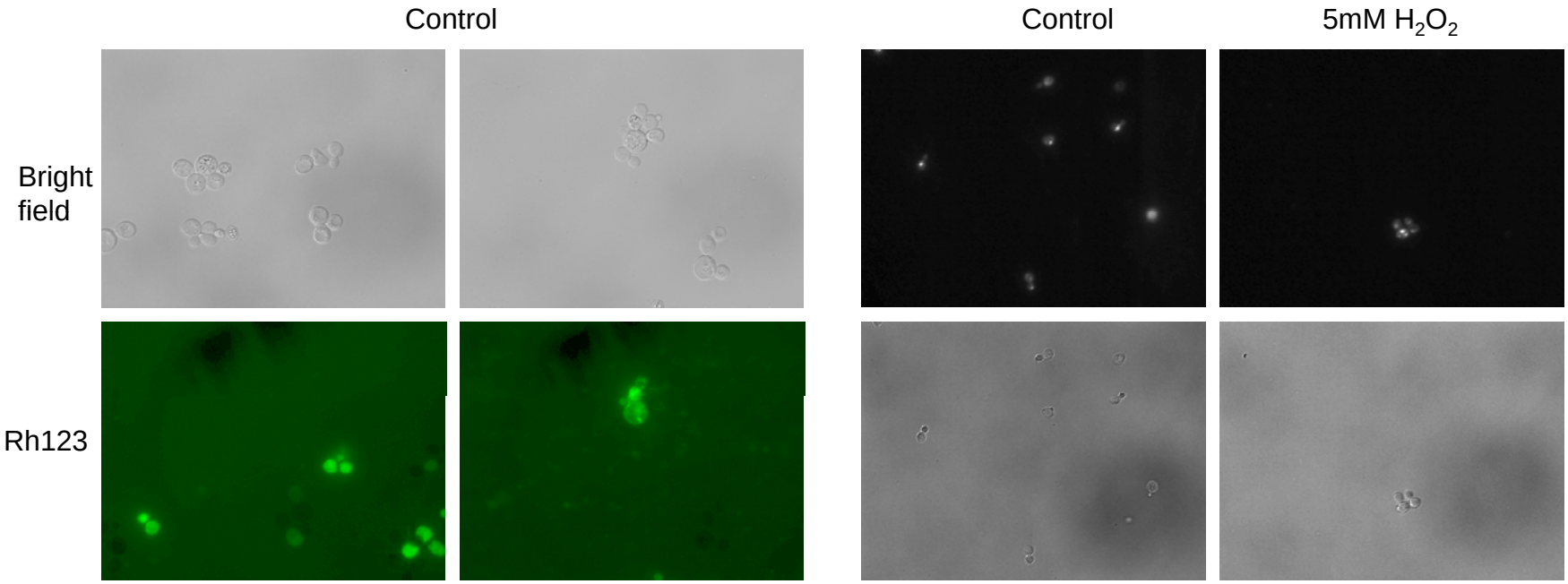


Fig. 4.2.1e and f. Yeast cells showing MMP collapse and DNA fragmentation with response to H_2O_2

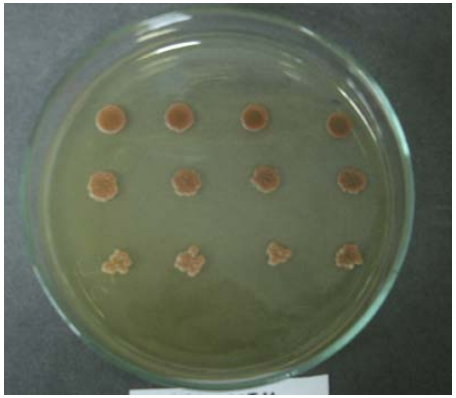
type, apoptotic characters like free cytosolic calcium rise, DNA degradation, MMP collapse (Fig 4.2.1a and 4.2.1 b), death percentage etc were analyzed.

After 60 minutes of H_2O_2 treatment, the cells were stained with propidium iodide (PI, 1μg/ml) and subjected to FACS and microscopy. Nearly 20-30% cells showed PI positive reaction (Fig 4.2.1c) and the same percentage of PI positive population were observed under Carl-Zeisis apotome (Fig 4.2.1d) and the H_2DCFDA preloaded cells were used to monitor the rise of total ROS upon induction of cell death with different stimuli. Cells treated with 5mM H_2O_2 showed the rise in green fluorescence intensity in FACS analysis, which could be due to the entry of external H_2O_2 into the cell and the cells also rise in green intensity under microscopy (Fig 4.2.1e). Cells incubated for four hours showed DNA fragmentation as compared to the control cells (Fig 4.2.1f).

The loss of mitochondrial membrane potential (MMP) is a key event in apoptotic cell death. This event has been well demonstrated in animals and plants against different death stimuli. Therefore, we examined H_2O_2 induced yeast population, to verify the above said MMP character. Yeast cells that were preloaded with Rhodamine 123 were subjected to H_2O_2 treatment and the loss of membrane potential was monitored by FACS. It showed clear reduction in mean green fluorescence by using FACS and by observing under fluorescence microscope (Fig 4.2.1f and h). These results were on par with already reported findings and hence H_2O_2 treatment was used as positive control in further experiments

4.2.2 Salt induces programmed cell death in yeast

The detrimental effects of high concentrations of salts (mainly Na^+) on plants remains a major limitation to agricultural productivity in arid, semi-arid and irrigated agriculture. Saline stress is one of the most common experienced abiotic stress and known to inhibit growth and time dependent reduction in cell viability.

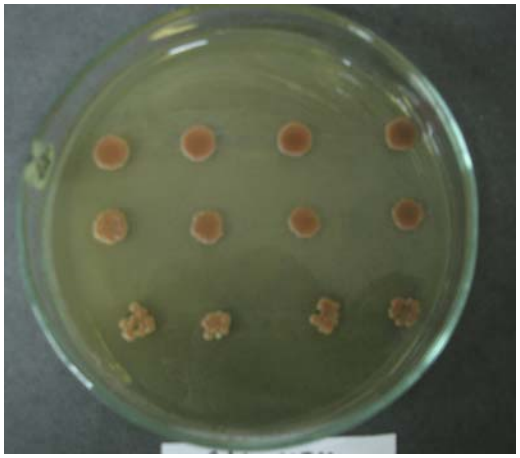


Sample at 2hrs

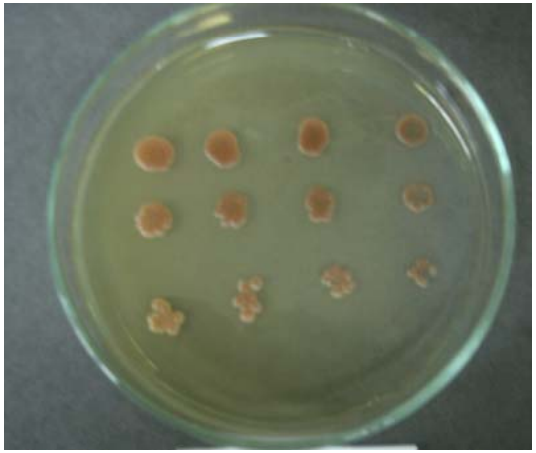
NaCl concentration

1.5M	1.0M	0.5M	0M	
				10^{-1}
				10^{-2}
				10^{-3}

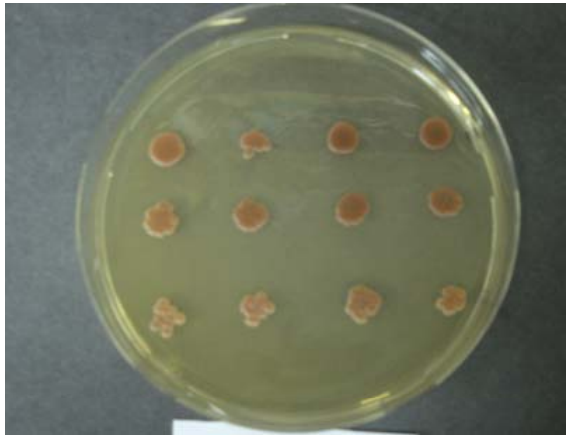
DILUTIOn



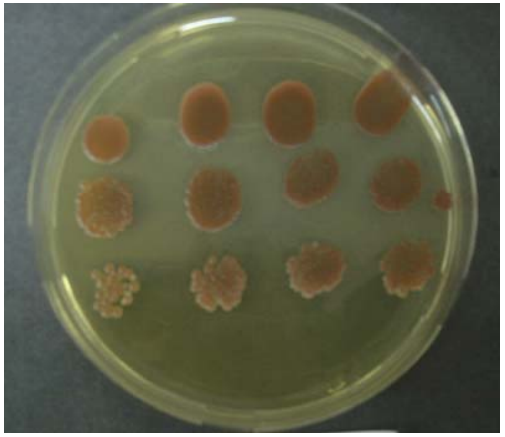
Sample at 4hrs



Sample at 0hrs



Sample at 6hrs



Sample at 8hrs

Fig. 4.2.2a.
Effect of Salinity on yeast growth

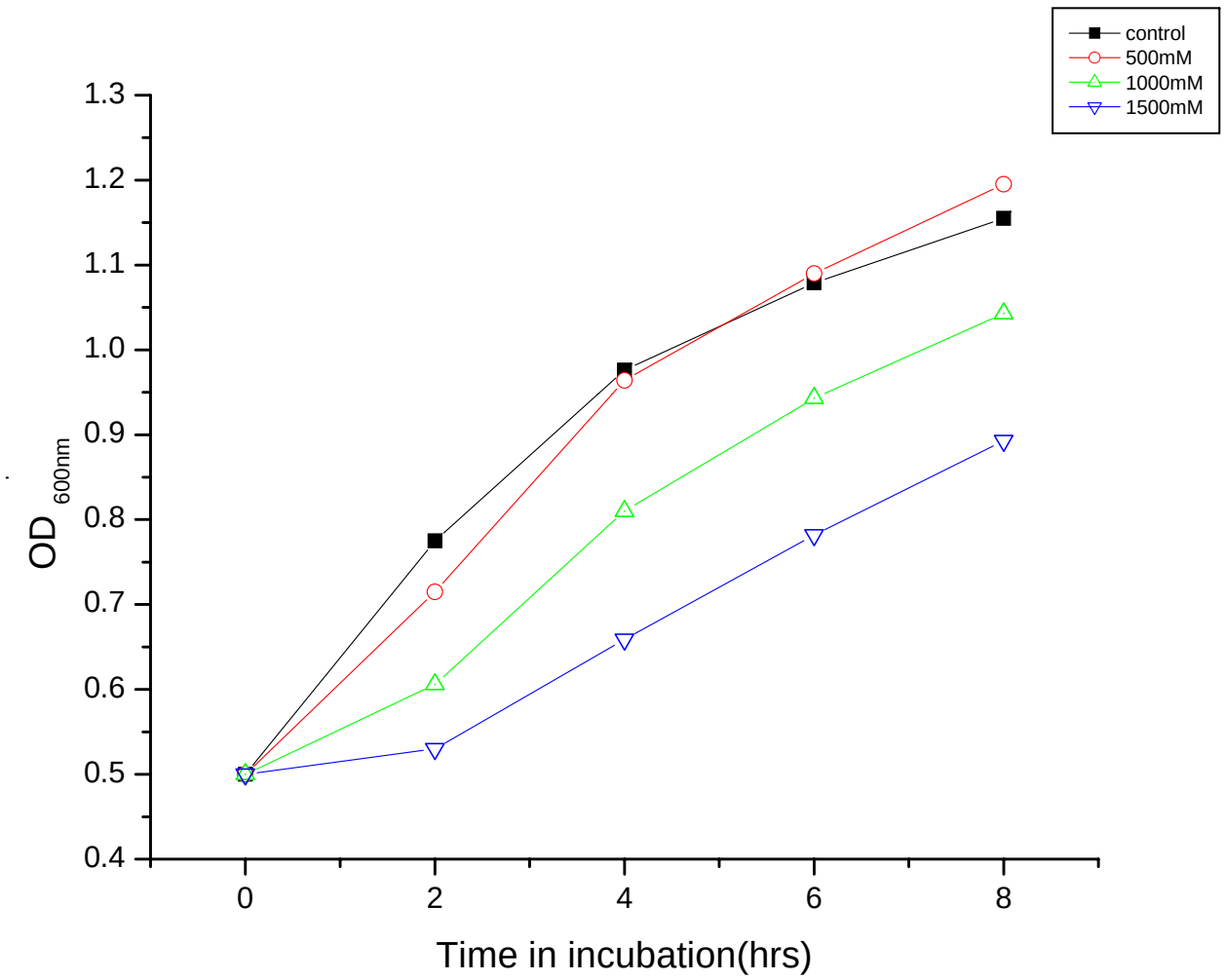


Fig. 4.2.2b. Salinity concentration and yeast cells death response curve

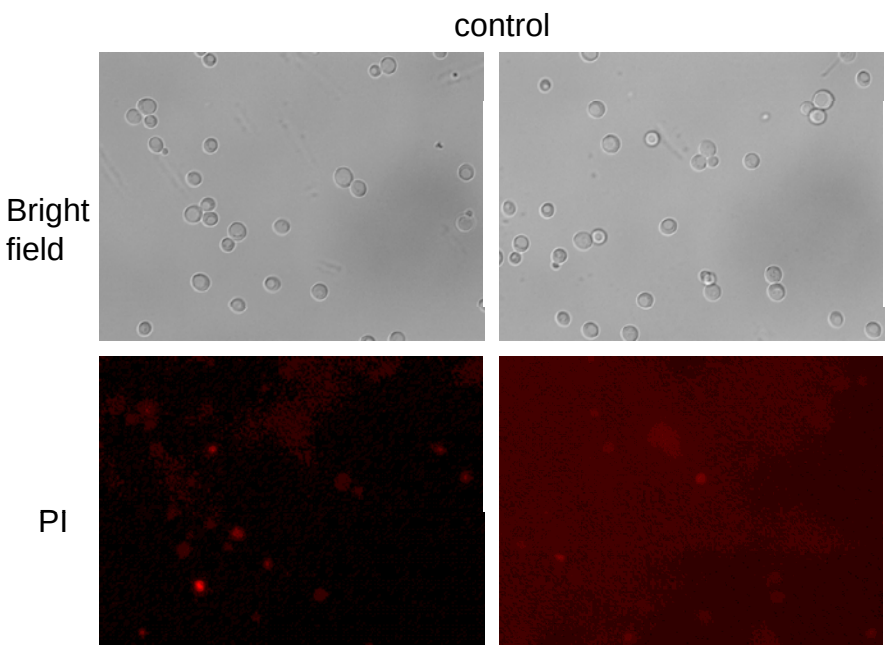


Fig 4.2.2c-b

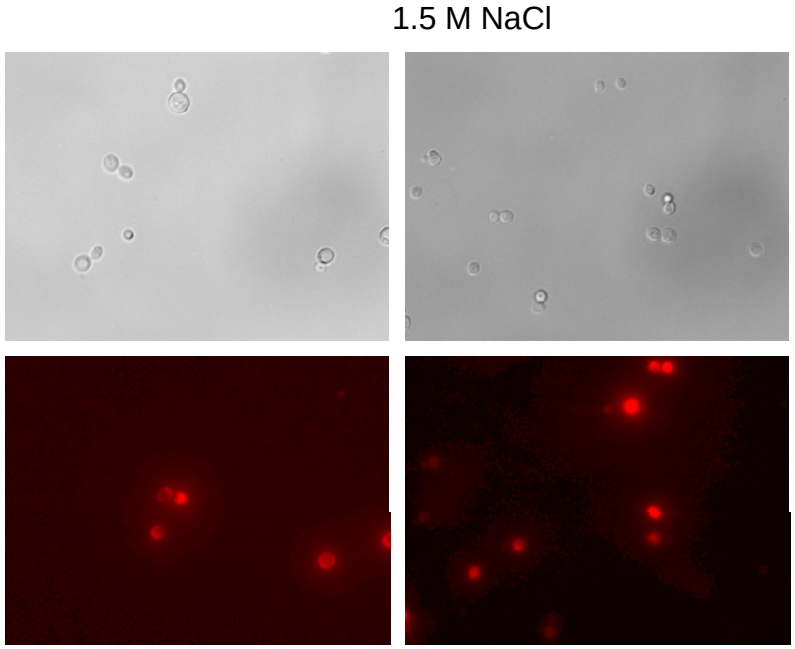


Fig 4.2.2c-c

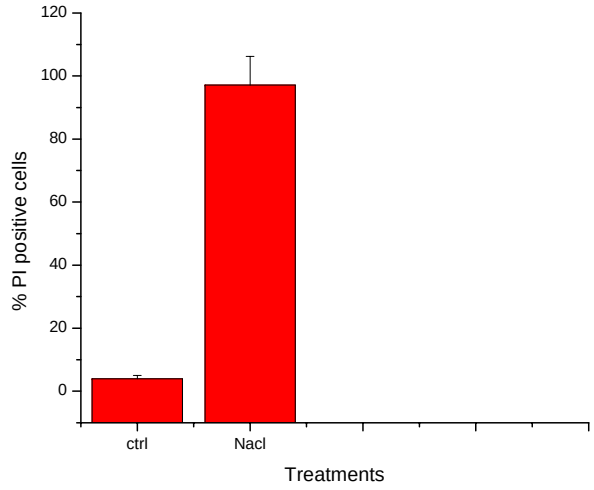


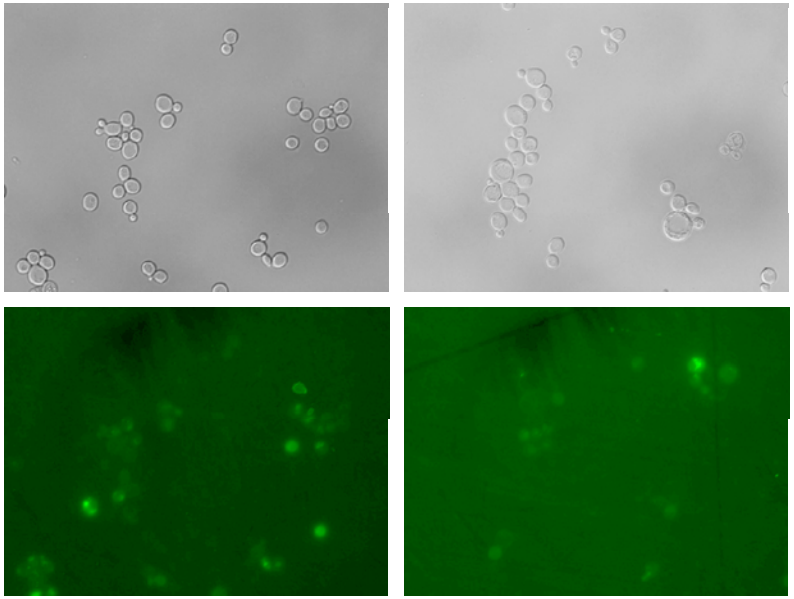
Fig 4.2.2d

NaCl treated cells stained with PI

b) Control

c) NaCl treated cells

d) FACS Data



Nacl+Rh123.1

Fig. 4.2.2e. Yeast cells showing MMP collapse with response to NaCl

Currently, the use of molecular genetic systems has become an increasingly important tool in understanding basic cellular functions. Here the yeast, *Saccharomyces cerevisiae*, has been used as a molecular genetic system to induce cell death by using NaCl treatment and score apopototic cell death parameters.

Therefore, another important stimulant like NaCl stress was used to induce the cell death in comparison with H_2O_2 as a stimulant in our experiments in yeast system. The cell growth inhibition was observed at concentrations above 1M NaCl treated exponential or mid log phase cells and the highest growth arrest was observed in cells treated with 1.5M NaCl (Fig 4.2.1a and 4.2.1b) and these results explained growth inhibition or cell division arrest that takes place in saline treated cells.

The growth curves were plotted with respect to NaCl treatment the results are presented as below. The maximum growth inhibition was observed in 1.5M NaCl treatment and there is no growth inhibition observed in control and 200mM NaCl treatments and therefore, further experiments are conducted by using 1.5M NaCl treatment. Death percentage is monitored by staining with PI and analyzed using FACS. These results showed that nearly 70-80 % cells positive to PI (Fig 4.2.1c) and the same percentage of PI positive population was observed under microscope in 1.5M NaCl treated cells (Fig 4.2.1d).

The addition of 1.5M NaCl showed no rise of ROS levels in yeast (Fig 4.1.1e). But after four hours, incubated cells showed DNA fragmentation as compared to the control cells (Fig 4.2.1f), which is very important result observed in NaCl treatment. The important apoptotic character, MMP loss is also observed in 1.5M salinity stressed cells. Therefore saline induces apoptotic type of cell death and is not mediated by the ROS, but may be mediated by calcium signaling and therefore, further experiments are conducted by pre-incubating yeast cells

with calcium and ROS scavengers. These experiments results discussed with effect of EGTA on Yeast cell death.

4.2.3 Heat induces programmed cell death in yeast

Heat is one of the well-characterized apoptotic stimuli in animal and plant system. Therefore, heat stimuli were used to induce apoptotic process in yeast system. The mid -log phase cells were exposed to 45^{o}C, 55^{o}C and 65^{o}C temperatures of each 10 minutes, the growth inhibition or cell division arrest was observed at 55^{o}C and 65^{o}C temperatures as compared to control (Fig 4.2.3a and 4.2.3b). Further, exposure of cells for 10 minutes at 55^{o}C was used for studying PCD characteristics.

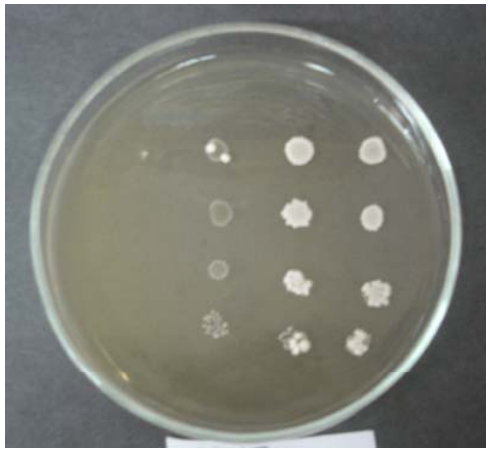
The growth curves were constructed by using OD 600nm values which were recorded at every two hours and the results as follows. The 65^{o}C and 55^{o}C heat treated cells showed complete inhibition in cell growth. The 55^{o}C treated cells were 89% PI positive (Fig 4.2.3c) as compared to the control and the same percentage of PI positive cells were observed under microscope (Fig 4.2.3d). In 55^{o}C treated cells, rise in the ROS levels was observed. As noticed with H_2O_2 treated and saline treated cells, DNA fragmentation was observed even in heat-treated cells (Fig 4.2.3f). Therefore it was concluded that heat treatment also induced apoptotic type of cell death similar to the one caused by H_2O_2 and saline.

4.2.4 Mannitol does not induce cell death in yeast

Mannitol is well known osmotic agent used in sphaeroplast preparation at lower concentration in yeast cells. Here we tried to see any impact of this on yeast cell death. Even at 5% concentration of mannitol treatment, yeast cells did not show any death or any rise in ROS or cytoplasmic free calcium in cells.

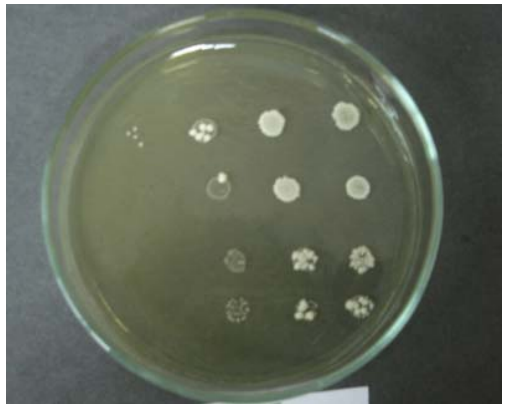
Therefore, upto 5% mannitol did not induce any death in yeast strain YPH 500.

65°C	55°C	45°C	C	
			10^{-1}	
			10^{-2}	dilution
			10^{-3}	
			10^{-4}	

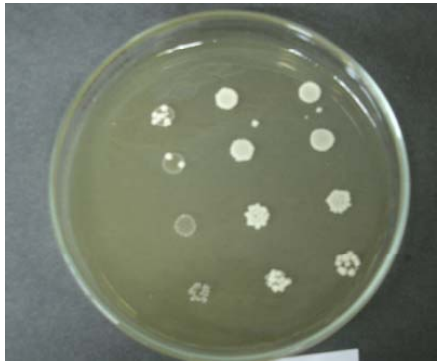


sampling after 2hrs

sampling after 0hrs



sampling after 4hrs



sampling after 6hrs

Fig 4.2.3a. Effect of heat on yeast cells growth

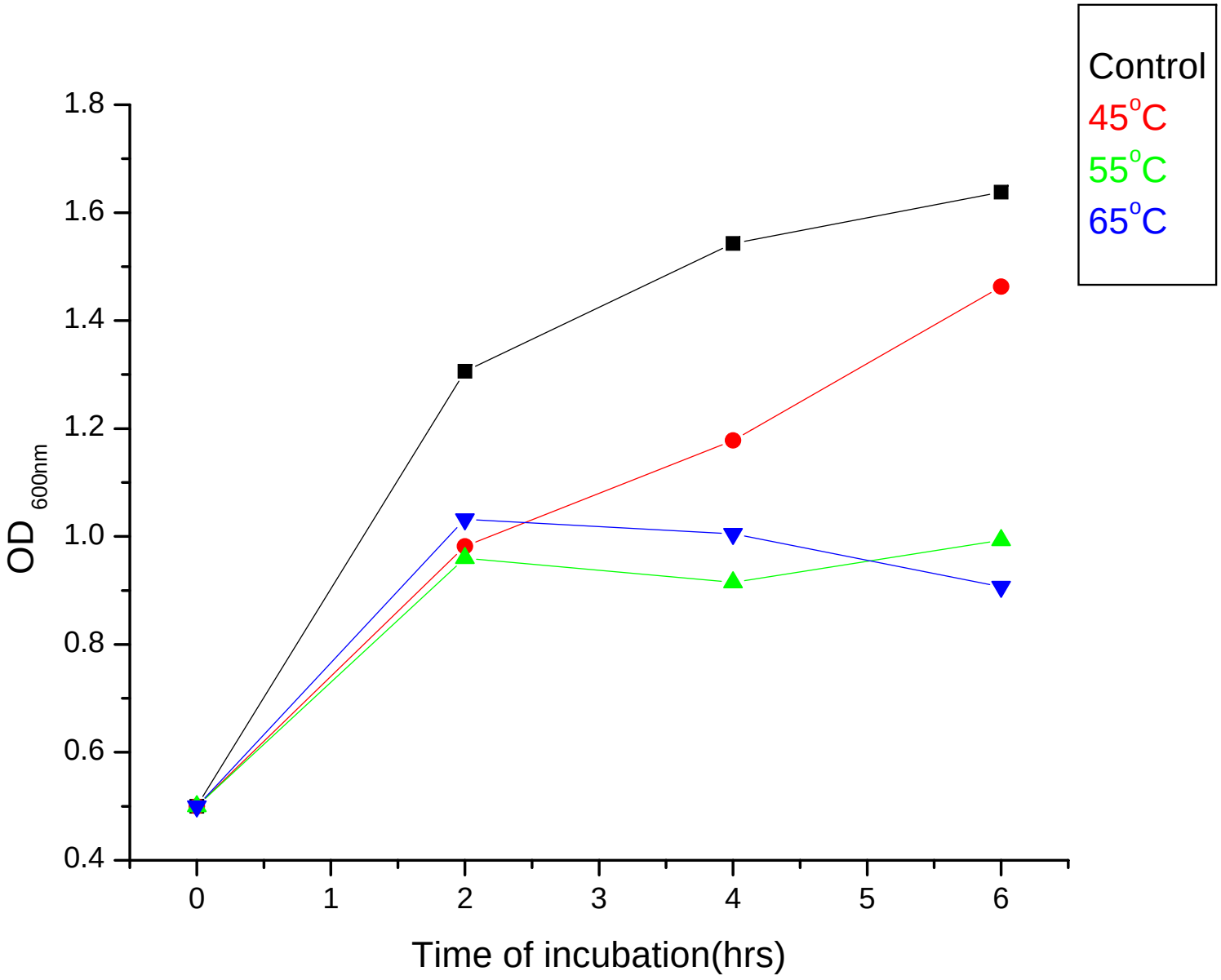


Fig 4.2.3b. Effect of heat on yeast cells growth response curve

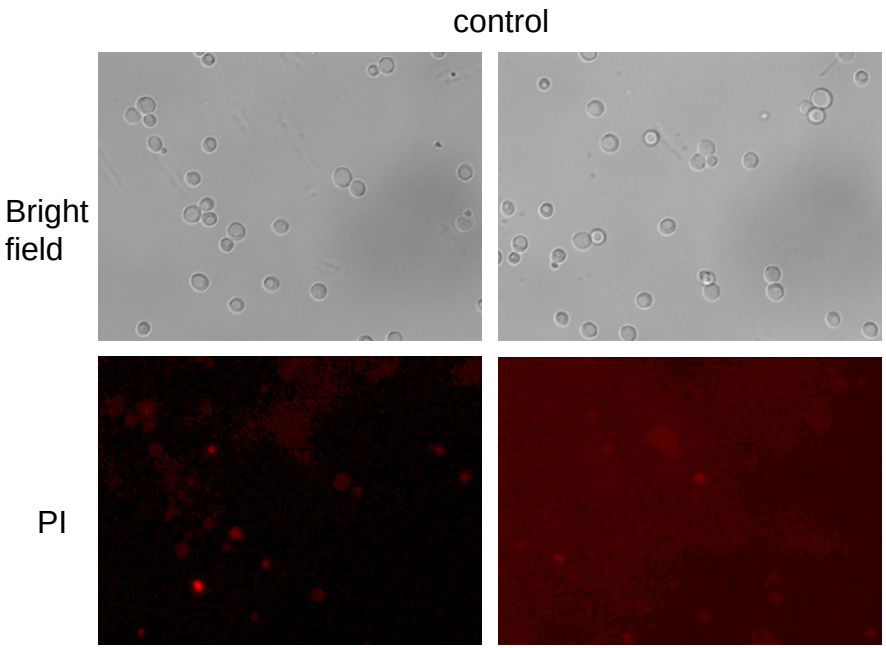


Fig 4.2.3c -b

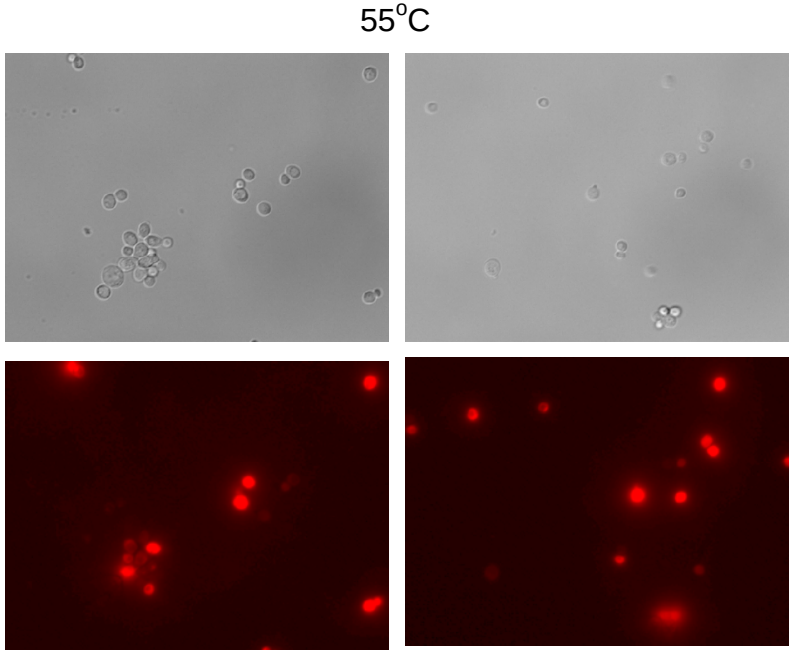


Fig 4.2.3c-c

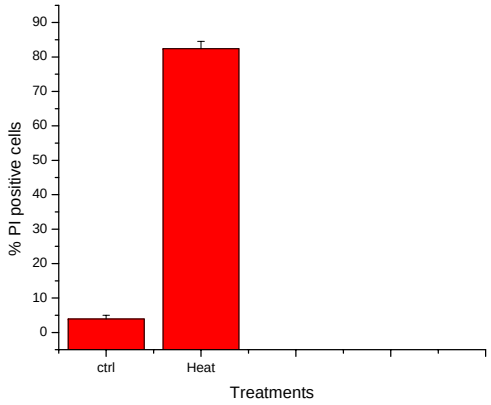


Fig 4.2.3d

Heat treated cells stained with PI

b) Control

c) heat treated cells

d) FACS Data

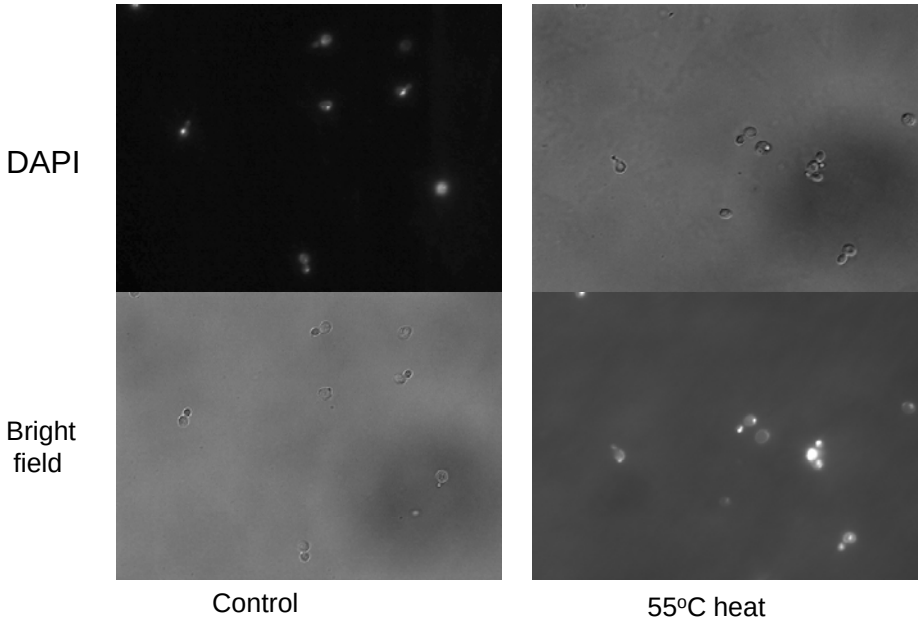


Fig 4.2.3e. Yeast cells with DNA fragmentation with response to heat stress

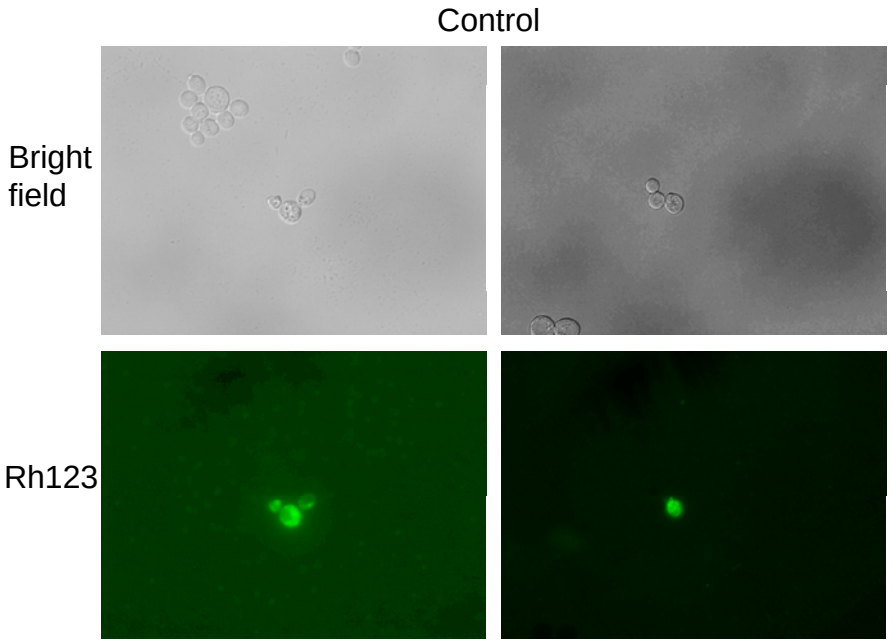


Fig 4.2.3f. Yeast cells showing MMP collapse to heat stress

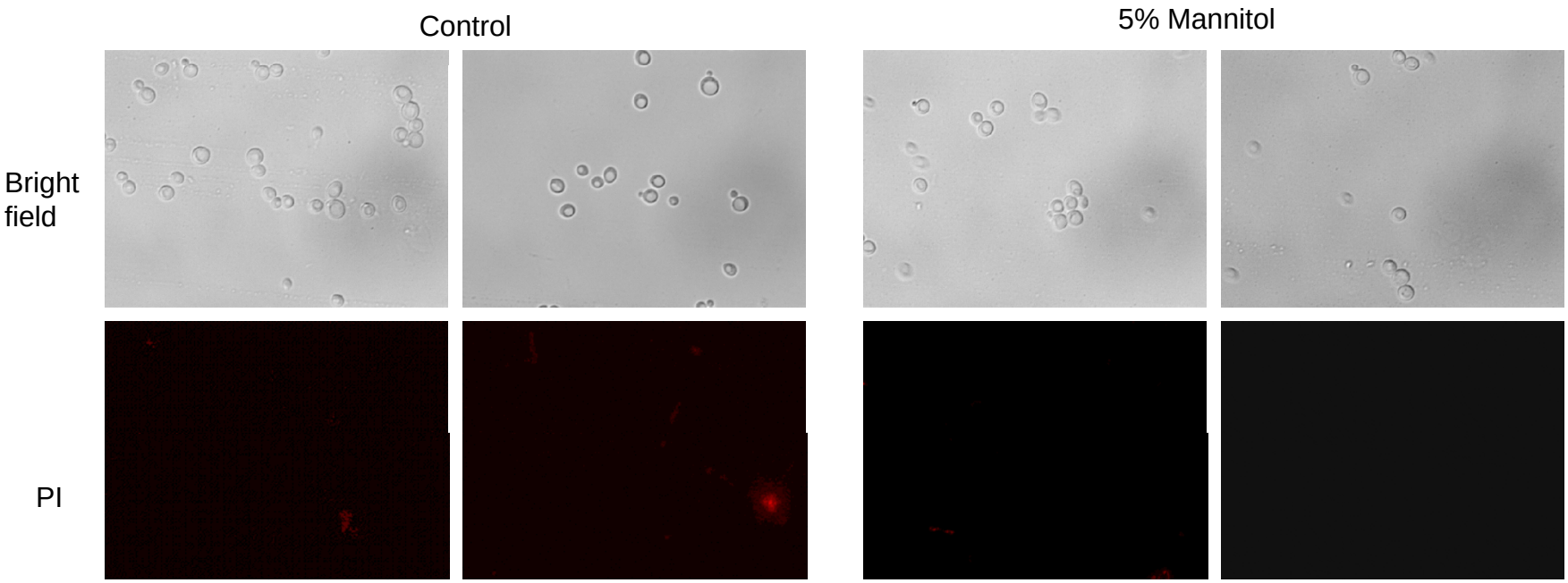


Fig4.2.4a

Fig4.2.4b

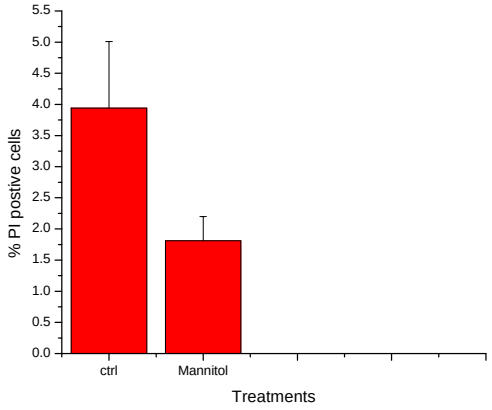


Fig4.2.4c

Mannitol treated cells are not show positive to PI

a) Control
b) 5 % Mannitol
c) FACS data

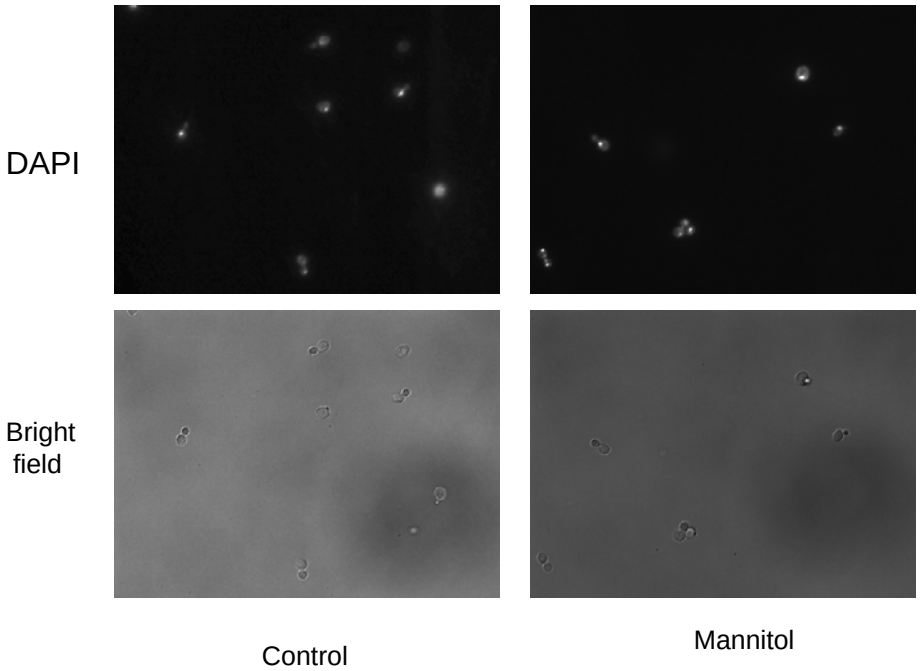


Fig. 4.2.4d. Yeast cells stained with DAPI with response to Mannitol

4.3 Yeast Transformation with Apoaequorin gene and analysis of cytosolic calcium levels during apoptosis.

Calcium is a well-known secondary messenger in animals, plants and yeast systems. Many intracellular and extracellular factors induce cytosolic free calcium rise resulting in a variety of physiological responses in animal systems such as proliferation, muscle contraction. The change of free cytosolic calcium plays a key role in regulating apoptosis in animal and plant cells and it was implicated as an early event in apoptosis (Budd and Nicholas., 1996). Therefore, yeast strain YPH 500 transformed with aequorin gene to analyze cytosolic free calcium levels against H_2O_2 and salinity.

The cytosolic free calcium rise was observed in both 5mM H_2O_2 treated and 1.5M NaCl treated cells as compared to the control cells (Data showed with effect of EGTA and TEMPOL on Cytosolic calcium rise). The cytosolic free calcium rise kinetics varied between H_2O_2 and NaCl treatments. In the case of H_2O_2 treatment, immediate and transient rise of cytosolic free calcium was observed, whereas in saline treatment, the rise was slow and steady (100seconds). With respect to amplitude, 600-700nm rise of cytosolic free calcium peak was observed in H_2O_2 treatment as compared to the control basal levels of 200nm (Fig 4.3a). In saline treatment, the amplitude was 1200nM as compared to the basal levels of 200nM in control cells (Fig 4.3b). This clearly showed that, in both cases cytosolic free calcium occurred at different time points after treatment and at different amplitudes.

Further, intracellular calcium levels were chelated by pre-incubation of cells with 10mM EGTA for about 60 minutes. In the EGTA pretreated cells, the rise of cytosolic free calcium reduction was observed in both cases of H_2O_2 (350μM calcium) and NaCl (810μM calcium) treatments as compared to the control cells. This experiment helped us to understand the reduced levels of

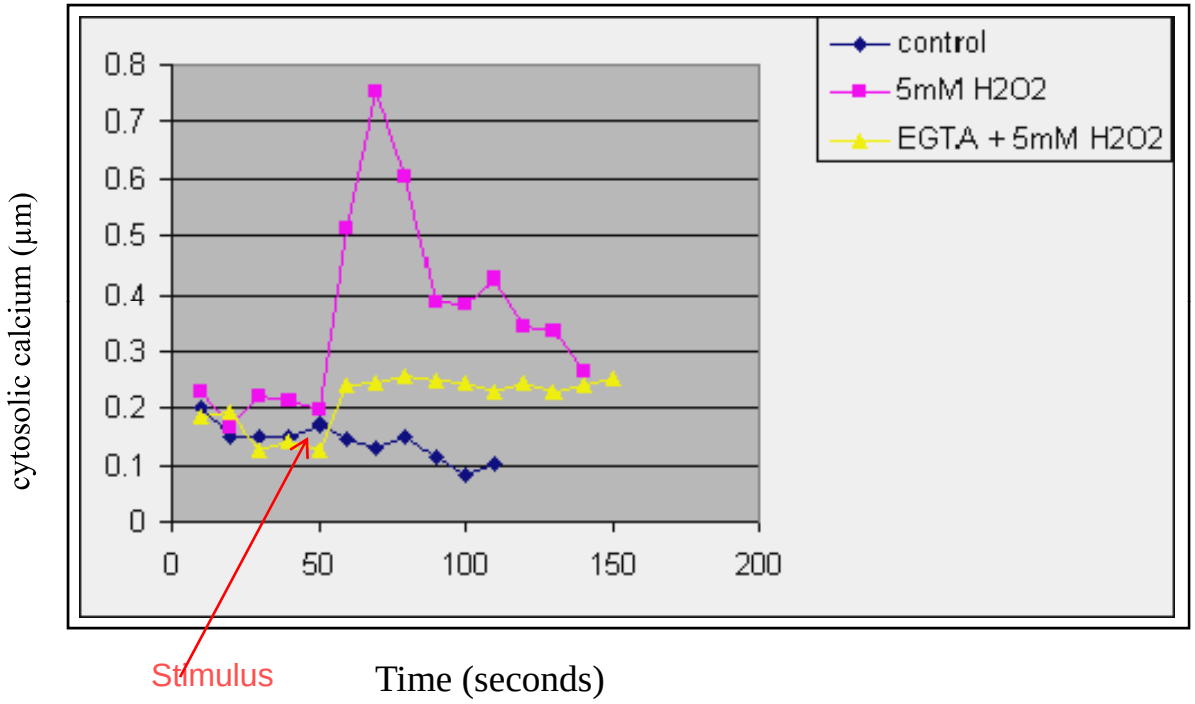


Fig. 4.3a. Effect of EGTA on H_2O_2 induced cytosolic calcium rise

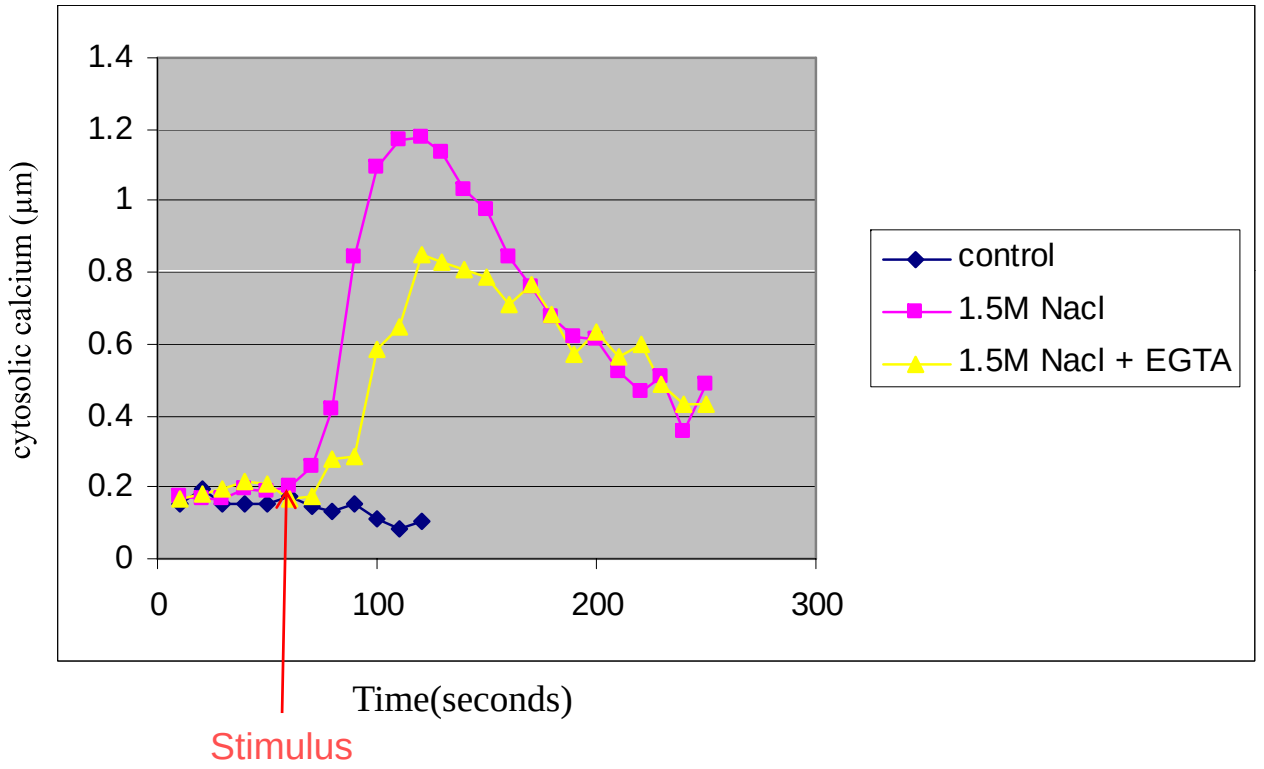


Fig. 4.3b. Effect of EGTA on NaCl induced cytosolic calcium rise

cytosolic free calcium during stress conditions and its impact on apoptotic death process.

Therefore, 10mM EGTA pretreated cells were further subjected to induction of apoptosis and death percentage was analyzed by PI staining using FACS and the result shows the reduction of PI positive percentage in EGTA pre-incubated yeast cells.

4.4 Effect of TEMPOL and Glutathione (ROS scavenger) on yeast apoptosis

Several workers have reported the generation of ROS in yeast upon induction of apoptosis by various death stimulants. Few workers used H_2O_2 itself to induce PCD in yeast. Scavenging of ROS by external use of TEMPOL and Glutathione (antioxidants) has not been studied. Therefore, we planned to scavenge the ROS by using 10mM TEMPOL pre-incubated cells to induce death and the death percentage was analyzed using FACS.

Once H_2O_2 caused cell death and rise in intercellular H_2DCFDA green fluorescence intensity was observed in yeast cells, the presence of internal ROS was chelated by pre incubation of yeast cells for an hour with 10mM TEMPOL and 2.5mM glutathione. Later, these cells were exposed to 5mM H_2O_2. As expected, 10-12% reduction in PI positive cells was observed in pre-incubated cells as compared to the control cells, but there was no considerable difference among glutathione or TEMPOL pretreated cells in reducing the death percentage.

But, TEMPOL or Glutathione pretreated cells were not protected against saline stress, the reason could be due to saline stress which may not cause rise in ROS levels.

Even though, both glutathione and TEMPOL are scavengers for ROS, in the case of heat stress, glutathione pre treated cells showed 20 % more reduction

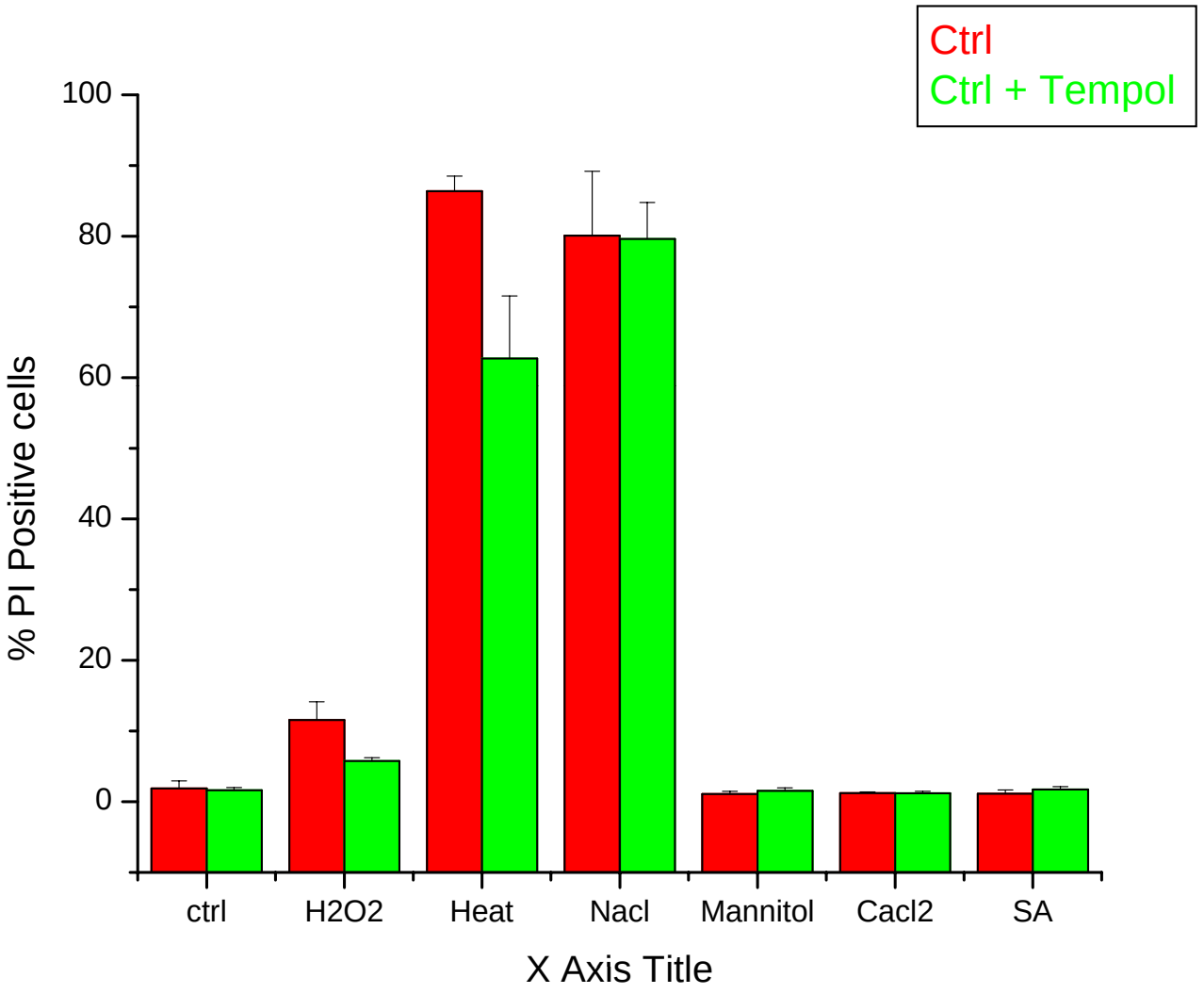


Fig. 4.4a. Effect of stimulants on Tempol pretreated yeast cells death

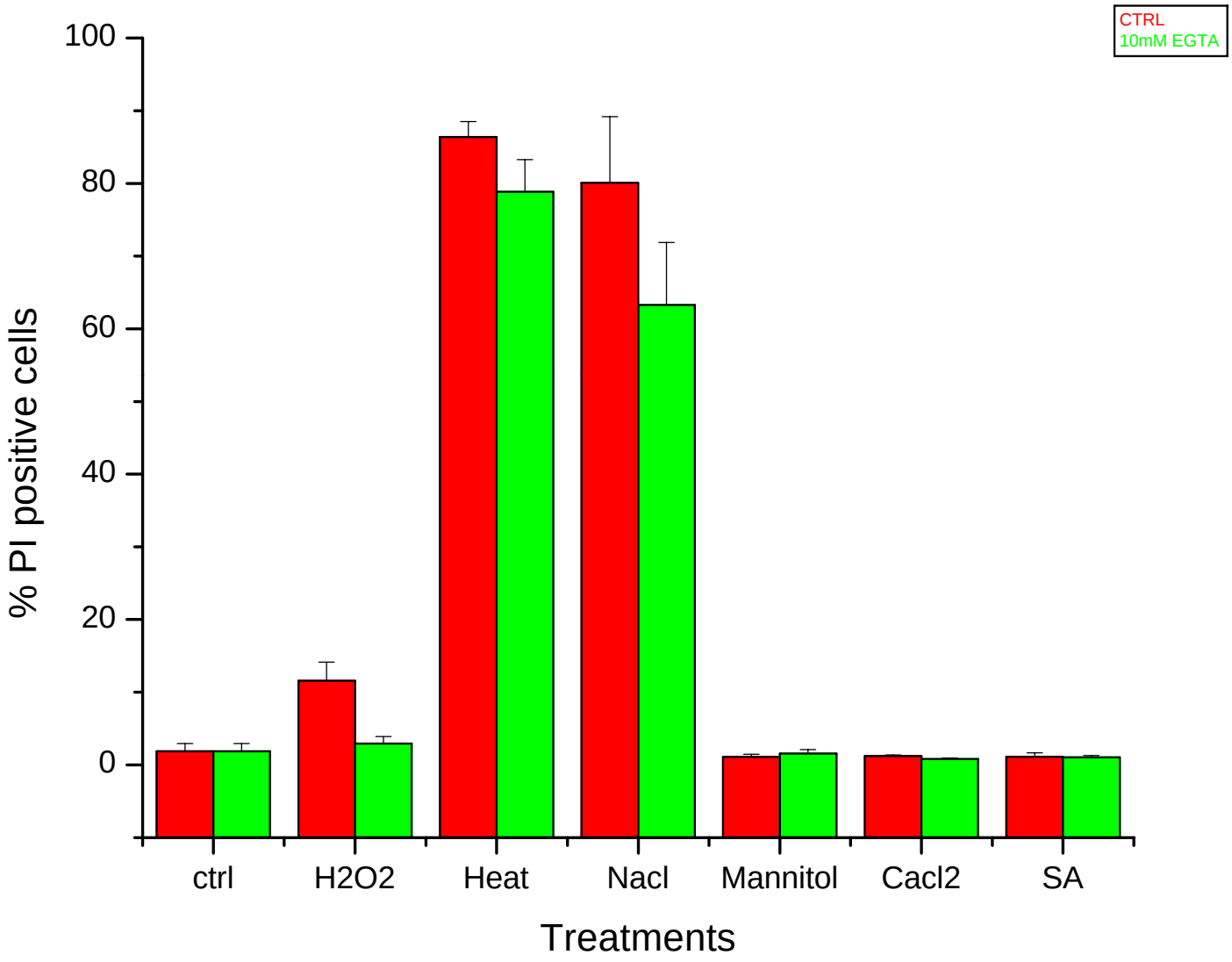


Fig. 4.4b. Effect of EGTA on death inducers on yeast cells

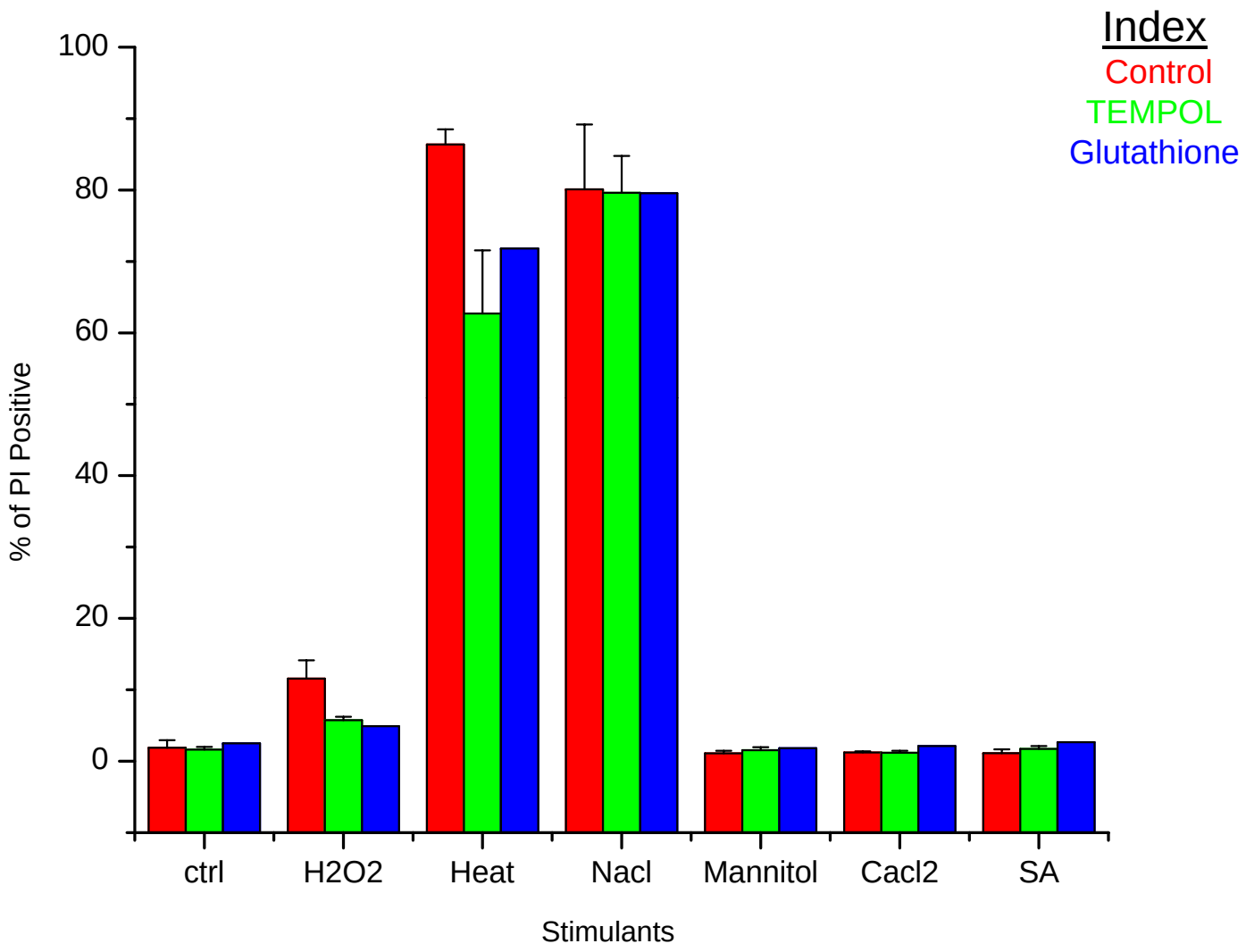


Fig.4.4c. Effect of stimulants on Glutathione and Tempol pretreated yeast cells

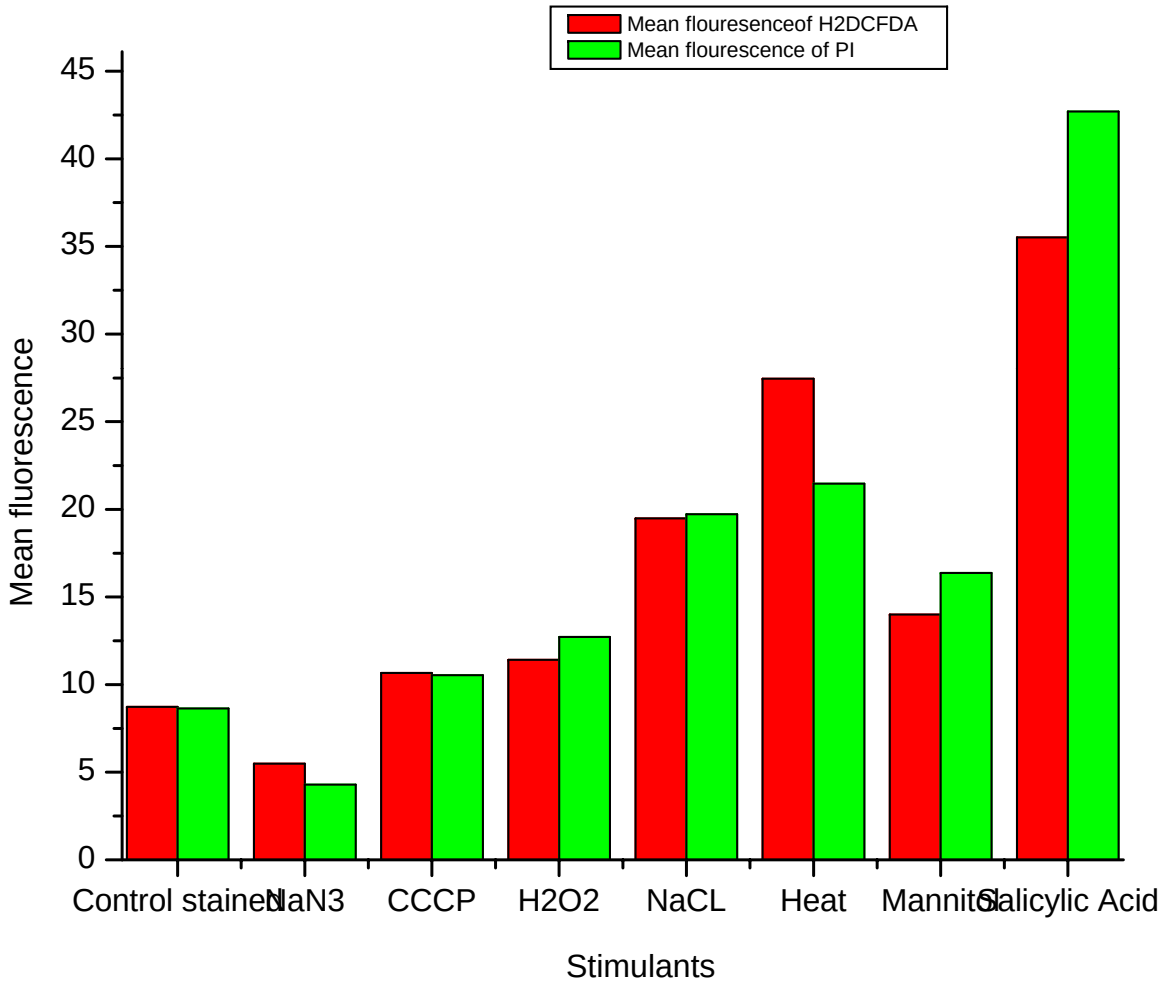


Fig. 4.4d. The rise of ROS against stimulants in yeast cells against stimulants

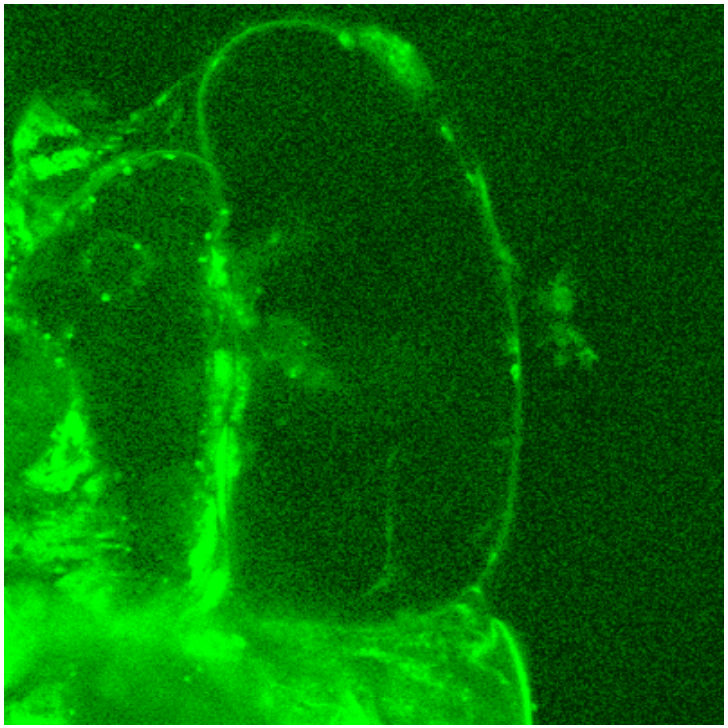
in PI positive cells than the TEMPOL pretreated cells. It indicates glutathione pre-incubation protects better than the TEMPOL pre incubation against heat stress. At the same time, pre incubation of cells with TEMPOL or glutathione did not cause any cytotoxicity (Fig.4.4a).

4.5 Effect of EGTA (Calcium scavenger) on yeast apoptosis.

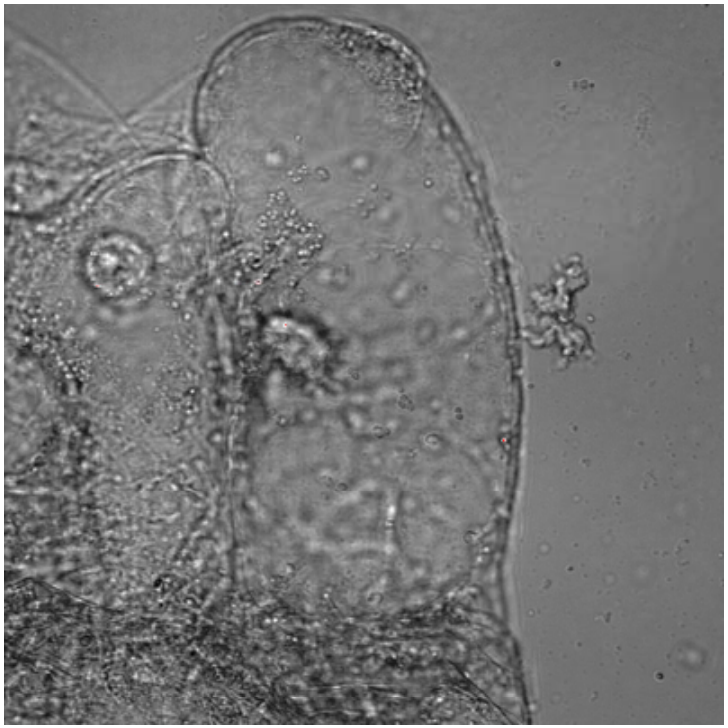
Halochim and Elilam(1993) reported that calcium functions as a ubiquitous intracellular messenger in animals and plant systems. The intracellular cytosolic free calcium rise was observed due to various abiotic and biotic stimuli and its further signaling transduction pathways. The EGTA is extensively used to scavenge intracellular free calcium in animal, plant and yeast systems. Therefore, yeast cells were pre-incubated with 10mM EGTA for about one hour and scored for death percentage by using PI staining against H_2O_2, salinity and heat treatments. 10-12% reduction in death was observed in the case of EGTA pre-treated cells against H_2O_2 stimuli, 40% reduction in the case of saline stress and 10-12% reduction observed in the case of heat stress. Highest reduction was observed under saline stress, where there was no ROS rise and no reduction of PI positive population against background of TEMPOL or glutathione pre-incubation and no cytotoxicity was observed due to EGTA pre- incubation (Graph 4.4b).

4.6 Transformation of tobacco cells transformation with At-vam5GFP, Bax-inhibitor-GFP and At-Fim-GFP

Three constructs At Bax inhibitor-GFP, AtVam5-GFP and fimbrin- GFP were transformed to a BY-2 cells using gene Gun. All three genes transformation was successful and the GFP expression was observed under microscope. These transformed cell lines will be used subsequently to study vacuole collapse, actin filaments dynamics (Fig 4.5a, b, c) etc. the transformation and GFP expression were observed. These cells will be possible to use future to study actin and vacuole dynamics during programmed cell death in BY-2 cell lines.

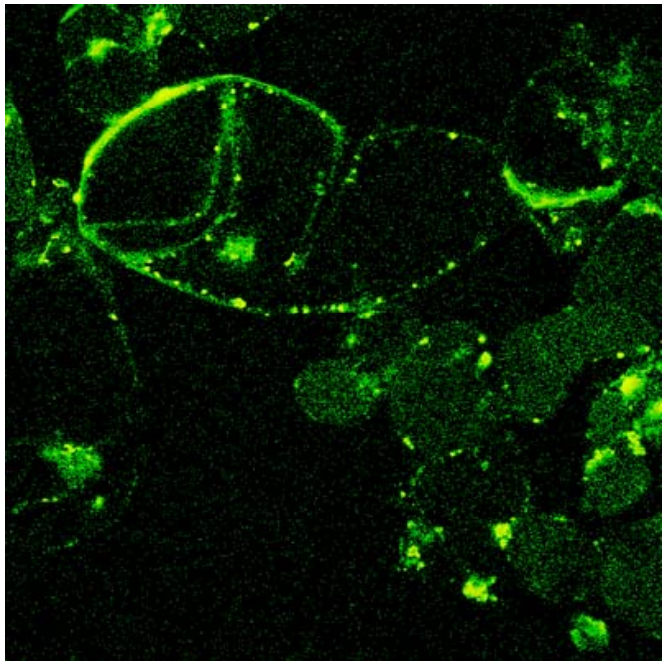


Fimbrin-GFP

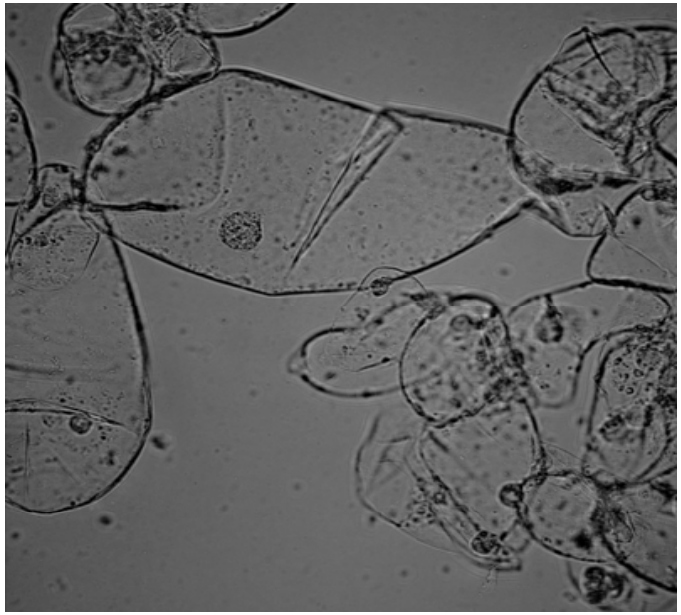


Fimbrin-GFP Bright field

Fig. 4.5c. BY- 2 transformants with Fimbrin-GFP

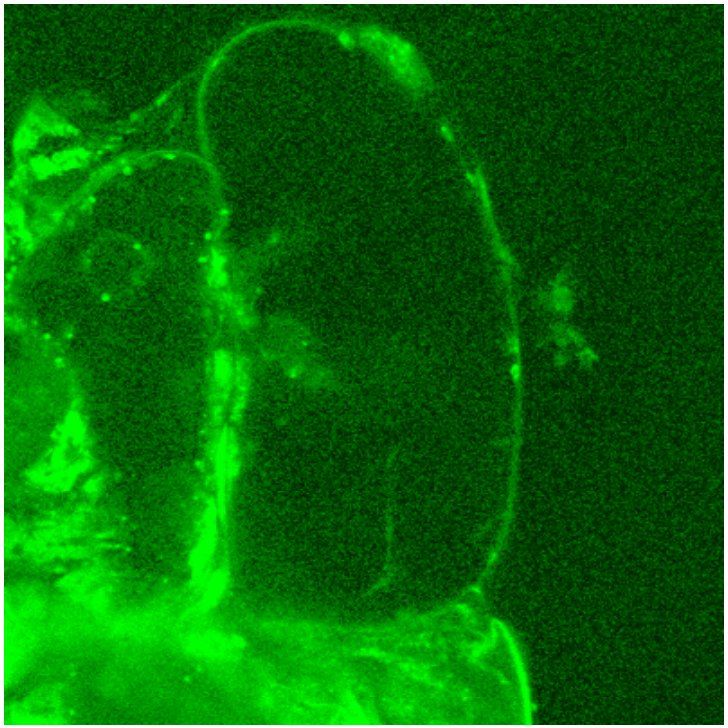


ATVam5 GFP

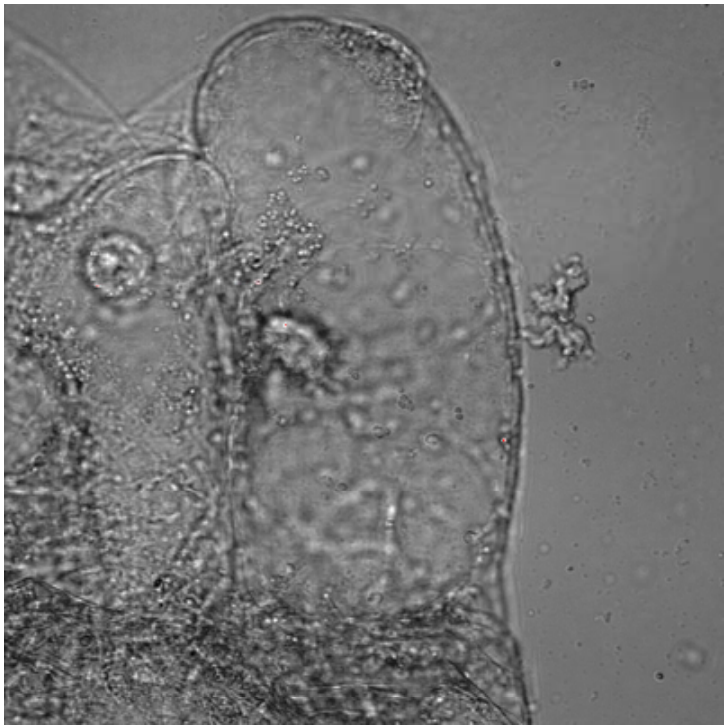


ATVam5 GFP-Bright field

Fig. 4.5b. BY- 2 transformants with *At* Vam5 -GFP



Fimbrin-GFP



Fimbrin-GFP Bright field

Fig. 4.5c. BY- 2 transformants with Fimbrin-GFP

V. DISCUSSION

Programmed cell death, the highly regulated destruction of cells occurs via a fundamental cellular process and is noticed in both unicellular and multicellular organisms. Its essentiality has been observed in maintenance of cellular homeostasis, regulation of development or metamorphosis or control of ageing. Across the kingdoms, same morphological and biochemical characteristics have been observed in the process of PCD. Many stimulants are known to result in PCD in animals. However, empirical data about the influence of these factors in inducing PCD in plants and yeast programmed cell death is not available. Therefore, few PCD stimulants known to induce PCD/ apoptosis in animal system were selected for observing their influence on PCD in yeast and plant systems. In our study the effect of H_2O_2, heat and salinity were assessed for their ability to induce PCD process.

In the initial experiments, H_2O_2, salinity and heat were used to induce death process in tobacco bright yellow-2 (BY-2) cell lines by studying the typical unique PCD characteristics. Later same stimulants were used to induce cell death in yeast system and the unique characteristics of PCD were studied. A comparison of these symptoms across animals like mammals, human cell lines, plant and yeast systems has been made. The results obtained during the course of investigations have been discussed here.

Reactive oxygen species (ROS) (considered as tissue damageing agent in connection with oxidative stress and ageing) and calcium are the important participants in signal transduction during apoptosis. In animal system, the rise of cytosolic calcium and ROS mediated signaling system is well elucidated by using EGTA and TEMPOL that act as scavengers of calcium and ROS respectively. Therefore, we used these scavengers to study the impact of scavenging cytosolic free calcium and ROS against well known death stimuli in yeast systems. These

experiments help to understand whether there is a link between cytosolic calcium and ROS during PCD process or they will act independently during PCD process. With these objectives, we conducted experiments in our laboratory and the results obtained are discussed.

5.1 Induction of PCD in tobacco cells (BY-2)

In this study, it was shown that exogenous application of H_2O_2 applied to BY-2 tobacco cell suspension cultures induced growth inhibition and PCD death process. The most important result obtained in this work was the observation that all major characteristics of PCD occurred following the treatments of 10mM H_2O_2. Cell content shrinkage with a high condensation of the cytoplasm, cleavage of nuclear DNA and vacuole collapse was observed. These results obtained support the following conclusion is "Exogenous H_2O_2 could initiate an PCD like cell death process in cultured tobacco BY-2 cells"

Similar to the data reported here, O'. Brien and co-workers (1998) showed that camptothecin, okaidic acid and salicylic acid or hydrogen peroxide and the calcium ionophore A123187 induced chromatin condensation and fragmentation in tobacco protoplasts (O'Brien *et al.*, 1998). Mittler *et al. (1998)* observed condensation and vacuolization of the cytoplasm and cleavage of nuclear DNA, during HR response of tobacco plants infected with TMV. The present study again showed that application of 10mM H_2O_2 to cultured BY-2 cells allowed the induction of cell death with characteristics of PCD as previously described in mammalian cells. Therefore, it could be concluded that certain aspects of PCD are conserved in both plants and animals. The concentration of H_2O_2 required to induce PCD in tobacco cells is quite high (10mM), as H_2O_2 was rapidly destroyed in plant cell cultures (due to presence of catalases and peroxidases) and the amount of H_2O_2 that penetrated into the cells was not easily measurable. 5-100mM of H_2O_2 was used earlier to induce cell death in plant cell suspension cultures

(Levine *et al.*, 1994; Desikan *et al.*, 1998). However, it was postulated that low levels of H_2O_2 may be sufficient for the induction of defense related genes, but higher concentrations are needed for the induction of cell death (Levine *et al.*, 1994). It is also known that the concentration of H_2O_2 required to induce PCD in tobacco BY-2 cells is higher than the concentration used to induce the apoptosis in yeast cells.

It is postulated that salt –induced PCD of *Arabidopsis* primary roots is a component of an adaptive response occurring *via* hypoxia condition created during stress. Salt stress is also believed to induce oxidative damage in plants. PCD is shown to occur in barley roots under salt stress (Katsuhara and Kawasaki, 1996). In our studies, it was observed that 200mM NaCl when applied to BY-2 suspension cultures was sufficient to inhibit growth and the cells show typical morphological and biochemical characteristics of PCD, when compared to H_2O_2 induced cell death. Therefore, from our experiments it could be concluded that

a) Salt stress induce cell death in BY-2 Tobacco cell lines at the lowest concentration of 200mM; and

b) Salt stress induced cell death show typical characteristics of PCD and vacuole disruption in tobacco protoplasts.

The salt stress induced hypoxia-mediated cell death is well reported in aerenchyma differentiation (Drew e*t al.*, 2000) in maize root cortical cells. Therefore, root cells exposure to salt stress condition helps in secondary root growth formation. The main difference between the H_2O_2-induced PCD and salt-induced PCD was the nature of vacuole collapse observed. In comparission to yeast, external application of 200mM NaCl was sufficient to induce PCD in BY-2 tobacco cells unlike in yeast, where it required nearly 1.5M NaCl. During our experiments, it was found that higher levels of salt stress causes cell death.

Heat stress has been shown to be one of the environmental factors that could trigger PCD in plant cells (Vacca *et al*., 2006; Zuppini *et al*., 2006). This process occurs with biochemical and morphological features similar to those observed in other animal and plant PCD pathways. Heat shock induced PCD is reported in carrot, *Arabidopsis* (McCabe and Leaver, 2000) and cucumber (Balk *et al*., 1999). In this study, we showed the effect of heat treatment on induction of cell death in tobacco BY-2 cells. Our results indicated that several PCD characters observed during PCD in higher eukaryotes can be identified in heat-induced cell death BY-2 cells. The presence of these key elements of the death molecular machinery that leads to typical morphological and biochemical features strengthen the idea of the existence of a conserved core set of components shared by multicellular organisms for induction of programmed cell death.

BY-2 cells exposed to H_2O_2, salinity or heat shock exhibited the typical PCD type morphological and biochemical markers. Among these hallmarks, the important character, which we have reported and was not shown previously, is the variation in the protoplast morphology and collapse of vacuole varies on exposure to the stimuli. In H_2O_2 treated protoplasts, clear cytoplasm condensation without loosing characteristic round shape of protoplast was observed. In heat-treated protoplasts, morphology varied between controls and H_2O_2 treated protoplasts. In H_2O_2 treated cells and protoplasts, granularity is highly condensed, but they will retain the complete shape without any breakdown. The salt stressed protoplasts were less condensed than the heat stressed but more than the H_2O_2 treated protoplasts. Salt stressed protoplasts were not necrotic type, because they retained the shape and did not burst. This type of morphological comparission is evident in apoptotic type of cells.

Later, we tried to transform tobacco BY-2 cells with apoaequorin gene construct. Transformants were obtained but expression levels are very low to

detect calcium signature. Further, we tried another method i.e. Flou-3 dye loading. Loading of dye is very poor and detection of calcium signature also becomes very difficult. Unfortunately, none of the experiments worked properly. Therefore, we decided to use yeast system to explore the relationship between ROS and calcium cross talk.

5.2 Induction of PCD in Yeast strain YPH 500

Apoptosis helps in homeostasis in higher organisms. In unicellular organisms like yeast, apoptosis mechanism has an evolutionary advantage. It appears more likely that the connection between nuclear condensation, DNA fragmentation, the inversion of the cytoplasmic membrane, and possibly the formation of cell fragments is evolutionarily old, and was used for the development of apoptosis by linking it to signaling pathways in metazoan organisms.

In mammals, exposition of phosphatidylserine is a earlier signal to undergo apoptosis (Fadok *et al*., 1992; Martin *et al*., 1995). This event has been reported in yeast exposed to various mammalian apoptotic stimuli. In mammalian and plant apoptosis, ROS are natural inducers of fatal cell damage, ageing, and cell death. A likely evolutionary mechanism for the development of apoptosis might be based on that phenomenon. ROS play a role in early and late steps of the apoptotic process, because ROS radical trapping prevents the cell death. It indicates ROS radicals act as signal molecules (Boggs *et al*., 1998).

However, ROS production prevention by actinomycin D, cycloheximide and caspase inhibitors Ac-YVAD-CMK showed inhibition of downstream events that follow transcription of caspase genes.

In our study, we found that external application of ROS member; H_2O_2 inhibits growth and leads to cell death. A few external stimuli like heat induces

ROS production initially and leads to death, suggesting that radicals are not just byproducts, but promoters of the apoptotic-like features in these cells. Yeast cell death triggered by low H_2O_2 concentrations with the phenotypic markers of apoptosis (Izawa *et al*., 1996) have been reported. These results illustrate a central role of ROS in apoptosis in yeast and which are agreeable with already published results.

In evolution, more complex regulatory pathways probably developed upstream of the basic mechanism, resulting in a complex signaling network. The phenomenon that glutathione is actively extruded during apoptosis of human monocytic cells (Ghibelli *et al*., 1995) may be a strategy to enhance ROS signaling- induced apoptosis. Some apoptotic pathways retain the usage of ROS in early regulatory steps; other pathways use it in later steps. We used TEMPOL and Glutathione as scavengers in our experiments to scavenge intracellular ROS levels and then study the effect of stimuli in inducing apoptosis in yeast.

NaCl stress is one of the environmental factors that has been demonstrated to induce apoptosis-like cell death in yeast and plants (Katsuhara, 1997; Huh *et al*., 2002). Wild-type cells of *S. cerevisiae* have been shown to exhibit apoptotic features after treatment with 1.2 M NaCl (Katsuhara, 1997; Huh *et al*., 2002). In our experiments, *S.cerevisiae* strain showed typical apoptotic characteristics after treatments with 1.5M NaCl. Therefore, our experiments further confirmed that the NaCl stress induced apoptosis in YPH 500 yeast strain. Further, the stimulus of NaCl was used to prove the link between ROS and calcium rise.

Heat stress is one of the major factors known to induce induces apoptosis in plant and animal cells. Lee *et al*, (2007) reported that temperature-sensitive yeast strain with *cit1* deletion, displayed a rapid loss in viability with typical apoptotic hallmarks, when exposed to heat stress and in our experiments, yeast cells exposed to 55^oC for 10 minutes showed the same type of apoptotic hallmarks.

Accumulation of ROS, loss of MMP was clearly observed. From our experiments with yeast cells and tobacco cell lines, it was established that these stimuli induced apoptosis in both the systems . However, there were differences with respect to levels of stimuli required for such condition. In case of yeast cells, lower concentration of H_2O_2 (5μM) was sufficient compared to 10mM required for tobacco cells. It was reverse for NaCl requirement with 200 mM NaCl being the concentration to induce apoptosis in tobacco cells, 1.5M NaCl is required for yeast cells. Therefore from the results, it was clear that H_2O_2, salinity and heat stress induced apoptosis in both yeast and tobacco cells.

5.3 ROS signaling during apoptosis in yeast

ROS includes superoxide radical, hydroxyl ions, singlet oxygen and hydrogen peroxide. Naturally ROS levels in cells are in equilibrium due to the balance between ROS production and scavenging mechanisms. The involvement of ROS in PCD was established on spatio-temporal correlation between increased ROS levels and cell death. Initially, Levine *et al*., (1994) showed that H_2O_2 induced cell death can be blocked by cyclohexamide and protease inhibitors and later it was shown in yeast mutant *cdc*48 by using H_2O_2 stimuli (Madeo *et al.*, 1999). Several workers have reported the generation of ROS in yeast upon induction of PCD by various death stimuli. Our experiments have shown the rise of ROS levels against salt stress and heat. Therefore, further experiments were conducted with ROS scavengers, TEMPOL and Glutathione, which have been used extensively in animal systems to scavenge ROS levels. Yeast cells pre incubated with TEMPOL or Glutathione showed reduction in cell death against H_2O_2 and heat treatments, but not against saline stress. Surprisingly, there was no rise in ROS against saline stress in yeast strain YPH 500 during our experiments. Similarly results have already been shown in animal and plant systems against H_2O_2. Our results have clearly shown the scavenging of ROS by TEMPOL /Glutathione being effective in yeast system against external H_2O_2 application and

internally generated ROS during heat stress. Also, our results have established that ROS is not required during salinity induced cell death in YPH500. Probably, the salinity induced cell death might involve another secondary messenger. Therefore, calcium might play role in saline stressed cell death in yeast strain YPH500. Therefore, further experiments were conducted to estimate cytosol free calcium in yeast often induction of PCD with H_2O_2 and saline stress.

5.4 Calcium signaling during H_2O_2 and saline stressed cell death.

Apoptosis, the active form of cell death occurs through various signal mechanisms, among those, loss of Ca^{2+} homeostasis control leads to apoptosis. In plant system, calcium is reported to be a secondary messenger in many signal transduction pathways that occur during heat shock, touch, anoxia, elicitor addition, pathogen infection, hormone administration, oxidative stress, far red light, drought, pollen tube elongation etc, (Knight *et al*., 1996, Subbaiah *et al*., 1994). Stephen *et al*., 2008, reported the rise of cytosol calcium in tobacco cells during osmotic shock and reduction of the cytosolic calcium rise by pre-incubation with EGTA.

The cytosolic calcium concentration in normal yeast cell is maintained in the range of 50-200nM and in the presence of biotic or abiotic stimuli, the calcium concentrations ranges from <1um to >100μM. In our results, both H_2O_2 and salinity induced transient calcium rise in yeast cells. The transient cytosol calcium rise is lowered with pre incubation of the yeast cells with 10mM EGTA as reported in plant cells. The calcium levels decrease observed both in H_2O_2 and saline treated cells. EGTA pre-incubated cells showed reduction in death percentage against H_2O_2, heat and salinity stimuli. This clearly indicated the role played by internal calcium flux under all the three stimuli in yeast cells. Significantly important results observed are that both TEMPOL and EGTA pre-incubated yeast cells showed reduced cell death percentage to external H_2O_2

application and at the same time calcium levels reduction was observed in cells pre-incubated with EGTA.

The yeast cells with saline induction showed transient rise in cytosol calcium and same set of experiments resulted in higher percentage of cell death. The EGTA pre-incubated yeast cells showed lowered calcium rise and lowered percentage of cell death. Where as TEMPOL / Glutathione pre-incubated cells did not show any reduction in cell death against saline stress. This might be due to non-involvement of ROS in saline stress induced cell death in yeast systems. Therefore, from the results obtained in the present investigation, the following conclusions were arrived at

- The PCD pathways occur in tobacco BY-2 cells and yeast YPH 5000 against the PCD inducing stimuli H_2O_2, heat and salinity
- The concentrations of H_2O_2 required for induction of PCD was higher in case of plants cells when compared to yeast cells
- The lower levels of salinity induced cell death process in plant system as against the higher concentrations required in yeast systems.
- In yeast and tobacco cell lines, upto 5% mannitol did not induce any cell death process.
- Cell death can be reduced by pre incubation of yeast cells with EGTA / TEMPOL / glutahione against H_2O_2 and heat.
- In saline treatment, only EGTA can reduce the cell death but not TEMPOL/Glutathione.

- The transient cytosol calcium rise was observed against both H_2O_2 and saline stress and the lowered transient calcium rise was observed in yeast cells pre-incubated EGTA.

- Thus, the ROS and calcium may mediate cell death independently against saline stress while both might be involved in H_2O_2 and heat stress induced cell death.

VI. SUMMARY

Plant cell biology studies have been done by using different plants cell cultures. the model systems employed in this study are tobacco (*Nicotina tobaccm*) Bright yellow-2(BY-2) cell line, because they are extensively used to conduct plant cell biology studies like programmed cell death, cytoskeletion, cell cycle regulation and cell growth and also gene discovery (*Geelen et al., 2001*). The use of BY-2 cells have unique advantages like highly synchronized growth rate and homogeneity, easy to maintain, transform and culture and bigger in cell size.

The budding yeast *Saccharomyces cerevisiae* is a unicellular, eukaryotic organism is widely used to understand various basic physiological processes. The similarities between yeast and mammalian system in comparison of subcellular structure and metabolic system are almost unique. We are also used this unique model system to conduct the programmed cell death studies in yeast.

In the initial experiments, H_2O_2, salinity and heat were used to induce death process in both tobacco bright yellow-2 (BY-2) cell lines and yeast to study the typical unique PCD characteristics and the results are summarized below.

Induction of PCD in tobacco cells (BY-2)

Few have reported that exogenous application of H_2O_2 to BY-2 tobacco cell suspension culture induces inhibition of growth and PCD death process. The present study again showed that application of 10mM H_2O_2 to BY-2 suspension cells induces cell death with characteristics of PCD as previously described in mammalian cells and BY-2 cell lines.

Again, our results showed that that the concentration of H_2O_2 required to induce PCD in tobacco BY-2 cells is higher than the concentration required to induce the apoptosis in yeast cells. i.e. 10mM in BY-2 cells compare to 5mM in yeast cells.

With respect to salinity, our studies, showed that 200mM NaCl is sufficient to BY-2 suspension tobacco cells to inhibit growth and stressed cells show typical morphological and biochemical characteristic of PCD type, where as in yeast, it requires 1.5M. Our results summarizes that, salt stress induce cell death in BY-2 Tobacco cell limes at lowest concentration of 200mM; and salt stress induced cell death show typical characteristics of PCD and vacuole disruption in tobacco protoplasts.

The main difference between the H_2O_2 and salt-induced PCD is the nature of vacuole collapse. In H_2O_2 stress, protoplast morphology were intact and internal contents are less shrinked, where as in Saline stress, protoplast morphology remains intact, but its internal content are more condensed than H_2O_2 stressed protoplasts.

In comparison to yeast salinity stress, external application of 200mM NaCl was sufficient to induce PCD in BY-2 tobacco cells, but in yeast, it required nearly 1.5M.

Heat shock induced PCD is reported in carrot, *Arabidopsis* and cucumber. In this study, we showed the effect of heat treatment on induction of cell death in tobacco BY-2 cells. Our results indicated that several PCD characters observed during PCD in higher eukaryotes can be identified in heat-induced cell death BY-2 cells.

BY-2 cells exposed to H_2O_2, salinity or heat shock exhibited the typically PCD type morphological and biochemical characteristics. In heat-treated protoplasts, morphology varied between controls and H_2O_2 treated.

Induction of PCD in Yeast strain YPH 500

ROS play a role in early and later stages of the apoptotic process, It indicates ROS radicals act as signal molecules (Hara *et al*., 1997; Boggs *et al*.,

1998; Tan *et al.*, 1998).In our study, we found that external application of ROS member, i.e. H_2O_2 inhibits growth and leads to cell death.

In case of yeast cells, lower concentration of H_2O_2 (5mM) was sufficient compared to 10mM required for tobacco cells. It was reverse for NaCl requirement with 200 mM NaCl being the concentration to induce apoptosis in tobacco cells, 1.5M NaCl is required for yeast cells. Our experimental results, it was clear that H_2O_2, salinity and heat stress induced apoptosis in both yeast and tobacco cells.

ROS signaling during apoptosis in yeast

In a biologically active condition, ROS levels are in equilibrium due to the balance between ROS production and scavenging mechanisms. Our experiments have shown the rise of ROS levels against salt, H_2O_2 and heat stress. Yeast cells pre incubated with TEMPOL or Glutathione (ROS scavengers) showed reduction in cell death against H_2O_2 and heat treatments, but not against saline stress. Our results have clearly shown the scavenging of ROS by TEMPOL /Glutathione being effective in yeast system against external H_2O_2 application and internally generated ROS during heat stress. Also, our results have established that ROS is not required during salinity induced cell death in YPH500. Probably, the salinity induced cell death might involve another secondary messenger. Therefore, calcium might play role in saline stressed cell death in yeast strain YPH500.

Calcium signaling during H_2O_2 and saline stressed cell death.

Apoptosis, the active form of cell death occurs through various signal mechanisms, among those, loss of Ca^{2+} homeostasis control leads to apoptosis. The rise of cytosol calcium in tobacco cells during osmotic shock was reported and the elevated calcium levels are reduced by pre-incubation of cells with EGTA (Calcium chelator).

In our results, both H_2O_2 and salinity induces transient calcium rise in yeast cells. The transient cytosol calcium rise is lowered with pre incubation of the yeast cells with 10mM EGTA as reported in plant cells. In the EGTA pre-incubated yeast cells, the lowered calcium levels were observed against H_2O_2 and saline treatments and EGTA pre-incubated cells showed reduction in death percentage against H_2O_2, heat and salinity stimuli.

The important results are that both TEMPOL and EGTA pre-incubation showed reduced cell death percentage to external H_2O_2 application and at the same time calcium levels reduction were observed in cells pre-incubated with EGTA.

The yeast cells with saline stress showed transient rise in cytosol calcium and higher percentage of cell death. The EGTA pre-incubation lowered both cytosolic calcium rise and percentage of cell death. Where as in TEMPOL / Glutathione pre-incubation condition, we did not observed any reduction in cell death against saline stress. This might be due to non-involvement of ROS in saline stress induced cell death in yeast systems.

Finally The PCD pathway occur in both tobacco BY-2 cell lines and yeast YPH 5000 against H_2O_2, heat and salinity. H_2O_2 required was higher in case of plants cells compared to yeast cells and concentration of salinity required in plant system is lower as against the higher concentrations in yeast systems.

In both yeast and tobacco cell lines, upto 5% mannitol did not induce any cell death process, In yeast system, Cell death can be reduced by pre incubation with ROS scavengers and Calcium chelator against H_2O_2 and heat, only EGTA can reduce the cell death against salinity but not ROS scavengers. Calcium rise was observed in both H_2O_2 and saline stress and transient calcium rise can be lowered by pre-incubation with EGTA.

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APPENDIX-I

Luria –Bertani broth (LB) pH 7.0

Tryptone - 10g

Yeast Extract - 5g

Sodium chloride- 10g

Double distill water- 1000ml

Adjust pH to 7.0

Yeast peptone Dextrose Medium (YPD) pH-6.9

Yeast extract- 10g

Peptone- 20g

Dextrose- 20g

Add to

Double distill water- 1000ml

Adjust to pH- 6.9

Synthetic complete (SC) medium pH6.9

MES- 117.5mg

Dropout mixture- 325mg

Yeast nitrogen base- 425mg

Ammonium sulphate- 1.250g

Glucose/dextrose- 5g

Amonoacids- 2075µl

Add to Double

Distill water to 250ml

Dropout mixture of amonoacids(5X)

L-Argenine- 240mg

L-Aspartic Acid- 1.2gm

L-Glutamic Acid- 1.2gm

L-Methionine- 240mg

L-Phenylalanine- 600mg

L-serine- 4.5mg

L-threionine- 2.4mg

L-Tyrosine- 360mg

L-valine- 1.8g

Liquid stock concentrations of Amino acids per100mL

Adenine sulphite-	500mg
L-histidine-	240mg
L-leucine-	720mg
L-lysine-	360mg
L-Tryptophan-	480mg
Uracil-	240mg

APPENDIX-II

1. Transformation of tobacco Bright yellow-2 cell lines with Atvam5-GFP, ATBI-GFP, Fimbrin-GFP, and Actin-GFP by Biolostic/Gene gun method.

Protocol for the gene gun (Iida et al.,1991)

i) Preparation of Micro Projectiles

1. Use 60 mg of tungsten powder (BioRad Tungsten M-17) for 100 shots.
2. Add 1 ml of freshly opened absolute ethanol.
3. Centrifuge for 10 sec in microfuge.
4. Replace ethanol. Add 1 ml water, resuspend tungsten and re centrifuge repeat 3 times.
5. Finally resuspend the tungsten particle 9n 1.0 ml of 50% glycerol.
6. Store the stock at -20^0C (use best before 3 months of preparation).

ii) Coating of plasmid DNA to microprojectile.

1. Vortex the microcarrier suspension prepared in 50% glycerol (60 mg/ml) for at least 5 min on a platform vortex to resuspend and disrupt agglomerated particles.
2. Remove 5µl of microcarrier suspension and put into a 1.5 ml microfuge tube. It is important to vortex the tubes containing the microcarrier continuously in order to maximize uniform sampling.
3. While vortexing vigorously, add in order:
    a. 5µl DNA (µg/ml),
    b. 50µl $CaCl_2$ (2.5M)
    c. 20µl spermidine (free base 0.1M)
4. Continue vortexing for 3 min.

5. Allow the microcarriers to settle for 3 min.
6. Pellet microcarrier by spinning 2 sec in microfuge.(Approx.5000 rpm).
7. Remove liquid and discard.
8. Add 150µl of 70% ethanol with out disturbing the pellet.
9. Remove liquid and discard.
10. Add 150µl of 100% ethanol.99% ethanol can also be used.
11. Remove liquid and discard.
12. Add 55-60µl of ethanol can also be used.
13. Resuspend the pellet by tapping the sides of the tube.
14. Remove 6µl aliquots of microcarriers and transfer them to the center of a macrocarrier.

iii) Preparation of tobacco BY-2 cells.

Approximately 10-15 callus, each about 0.5cm diameter, were sliced into smaller pieces (2-3mm) and placed in the middle of a plate containing Osmotic medium (MS basal medium, 0.5 M D-Mannitol, 30g/L sucrose, 2.4 D 2.5mg/L and 3g/L phytagel (Vain et al., 1993) for 4h prior to bombardment. The callus were left on medium overnight following the bombardment before being transferred to subculture medium.

B) Shooting of Tobacco Bright yellow-2 cell lines:

For each shot you will need 1100 p.s.i rupture disc (the small dark down discs), one flying disc (the large orange discs), and one stopping screen (the wire mesh screens). All these are supplied in the BioRad shooting kit. Before shooting, sterilize the number of discs and stopping screens that you will need by placing them for 5-10mins in a petri dish containing absolute ethanol. The discs and

screens are dried by standing them up along the side of a sterile petri dish and allowing them to air-dry, under sterile conditions in the tissue culture hood.

While sterilizing the disposable shooting components, the gun parts should also be surface sterilized by wiping with absolute ethanol. The components can be removed from the gun vacuum chamber for surface sterilization. These are the holder for platform for the leaf samples. After removing these and wiping with ethanol, the interior of the vacuum chamber and door should be wiped with absolute alcohol. Finally the five metal ring holders for the flying discs should also be surface sterilized.

C) Shooting procedure for PDS 1000/He Biolistic particle delivery system (BioRad Hercules, Calif.).

1. Turn the gun on (first red button on the left).

2. Press down the Macrocarriers discs with the red plastic"capplug", so that they fit snugly into the holders.

3. On the surface of each disc, pipette 5µl of DNA-coated tungsten. Spread microcarrier over the central 1cm of the macrocarrier, and dry the microcarries.

4. Place a rupture disc into the rupture disc holder, using forceps. Make sure the disc is properly seated in the holder by tapping the holder a few times gently. Screw the holder into the vacuum chamber and it must be screw in tightly.

5. Place a stopping screen onto the diaphragm in the flying disc holder assembly. On top of this place upside down a metal ring containing a flying disc with DNA onto it. Secure all the components by screwing on the assembly ring. Place the stage in the first slot from the top in the vacuum chamber.

6. Take a petriplate with callus and place it on the sample platform (lid Off). The sample platform should be positioned in the chamber 9cms distance between the stopping screen and target tissue, two shots per target plate.

7. Press the vacuum button (middle red button, up position) to begin pumping air out of the shooting chamber. Allow the vacuum pressure to reach at least 25inches

of Hg.Then press the shooting button continuosly (red button on the right, up position). Release the shooting button; remove the vacuum by setting the vacuum button to vent (middle position).

8. Take the petriplate out of the chamber. Incubate it in dark for 24-48hrs and then transfer it onto the selection medium.

"Improved" lithium acetate Yeast Transformation protocol

1. Grow 50 to100 ml cells to OD600~0.5(1-2 x 10000000 cells/ml).

2. Pellet cells, then wash with fresh 5ml Li-TE buffer.

3. Resuspend pelleted cells in 1 to 2ml Li-TE buffer.

4. Rotate 60 min at 30°C.

5. Place cells on ice for 10min.

6. For each transformation, mix in eppendorf tube: plasmid or cut DNA (in upto 10µl volume),1 µl salmon sperm carrier DNA (10mg/ml) and 100 µl cells.(Optional: leave 10 min at room temperature).

7. Add 100 µl 70% PEG, pipette and mix with blue tip (for P1000).Cut end off blue tip before using to widen bore.

8. Incubate at 30°C for 30 min.

9. Heat shock cells at 42°C for 15 min.

10. Spin at 1200 rpm,3 min. Aspirate PEG.Add 100µl of ddH_2O.Resuspend before plating plating on selective media.

Buffers:

10X TE is 0.1M Tris-HCL, 10mM EDTA, pH 7.5.

10X LiOAc is 1M LiOAc pH 7.5, adjusted with dilute acetic acid.

Make up TE and Li-TE from 10X stocks.

For PEG autoclave solid PEG 3500 or 4000 in TE.this will get it dissolved.PEG may precipitate out- warm at 37°C to redissolve.

2. Aequorin assay to calcium estimation

Grow starter cultures in selection media (SC,-ura)

↓

Inoculate into 25ml of media with selection and incubate till it reaches OD600nm-0.5

↓

Spin down and resuspend pellet into 1ml of SC-ura media and adjust OD600nm to 10.00

↓

Load the cells at the concentration of 3um coelentrazine per ml and incubate overnight under dark condition at $30^{o}C$

↓

Check the OD 600nm and adjust it to 10.0

↓

Second loading was done for 3.5hrs at a concentration of 48um per 50ul of cells and incubate the cells under dark condition

↓

Spin down and remove the supernatant and put it in the same volume of media.

Equilibration with calcium is done for 1hr if required

↓

Preincubation with inhibitors done for 15 minutes if required

↓

160ul of media + 10ul of cells are added to a cuvet and kept in Luminometer

↓

Record the intensity of luminescence at every 10 seconds. (As a Basal Value, Stress value and Lmax).