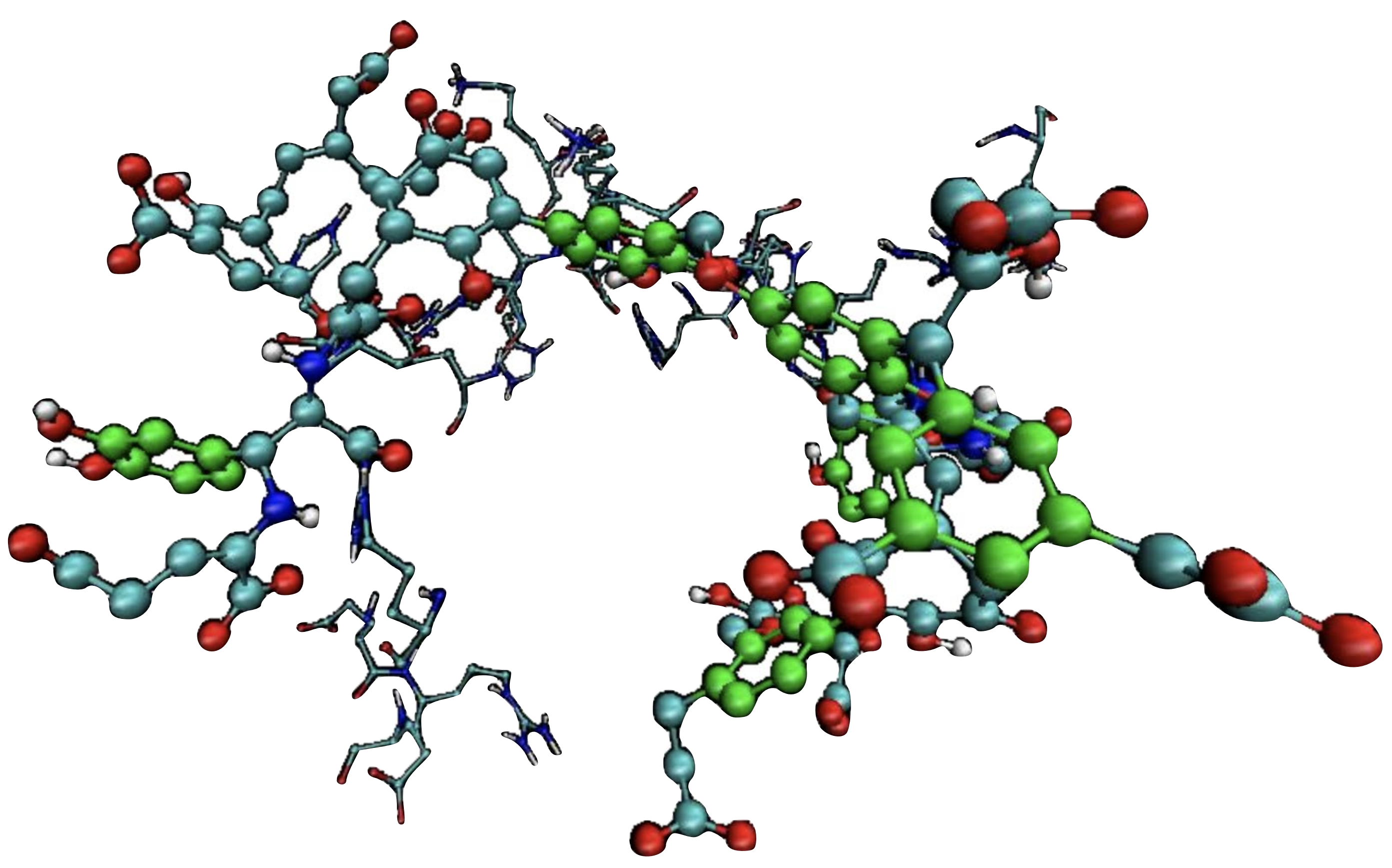
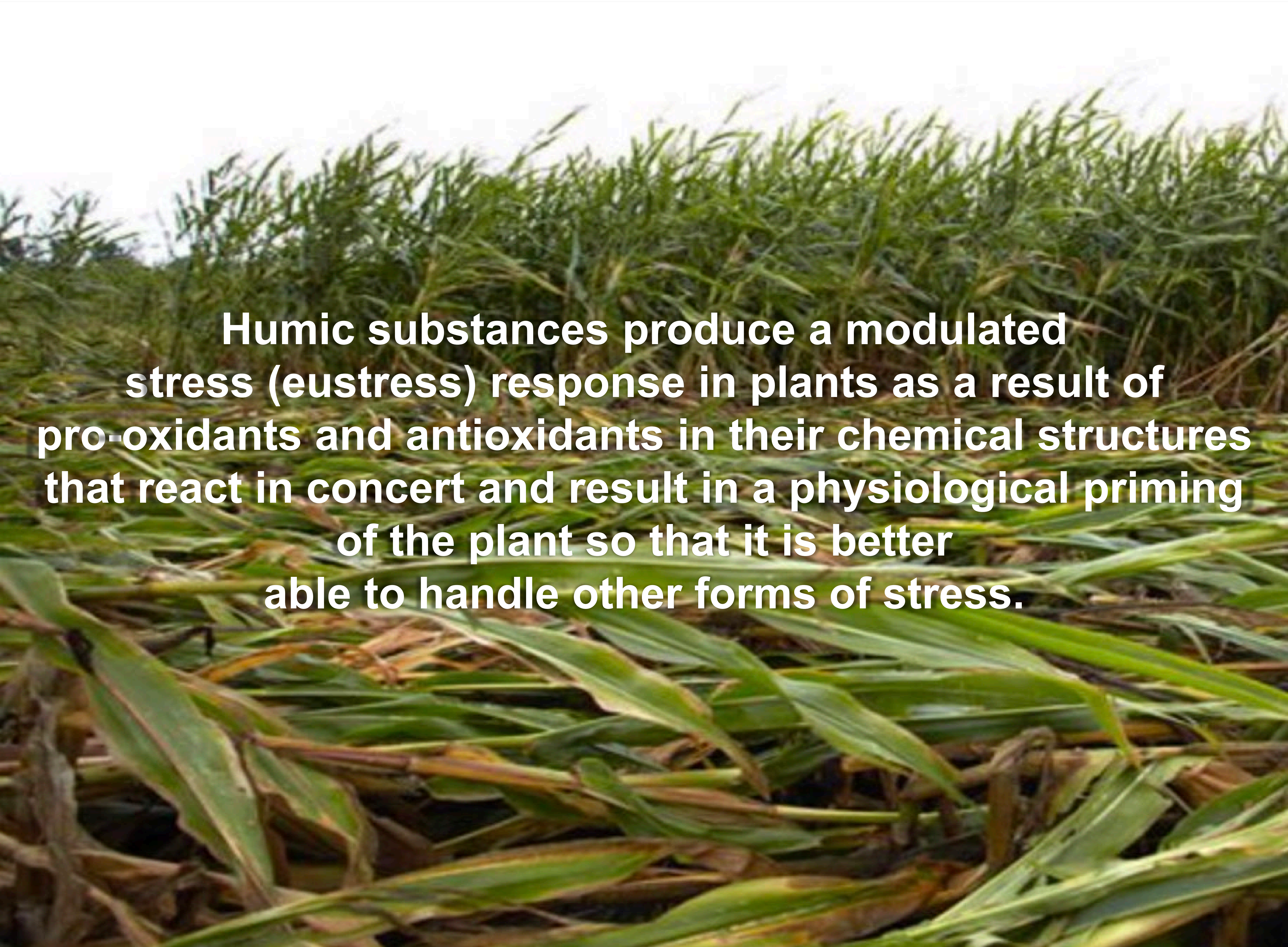
Unlocking the Enigma of Humic Substances Bioactivity: How Do Humic Substances Elicit a Beneficial Response in Plants???




Humic substances produce a modulated stress (eustress) response in plants as a result of pro-oxidants and antioxidants in their chemical structures that react in concert and result in a physiological priming of the plant so that it is better able to handle other forms of stress.

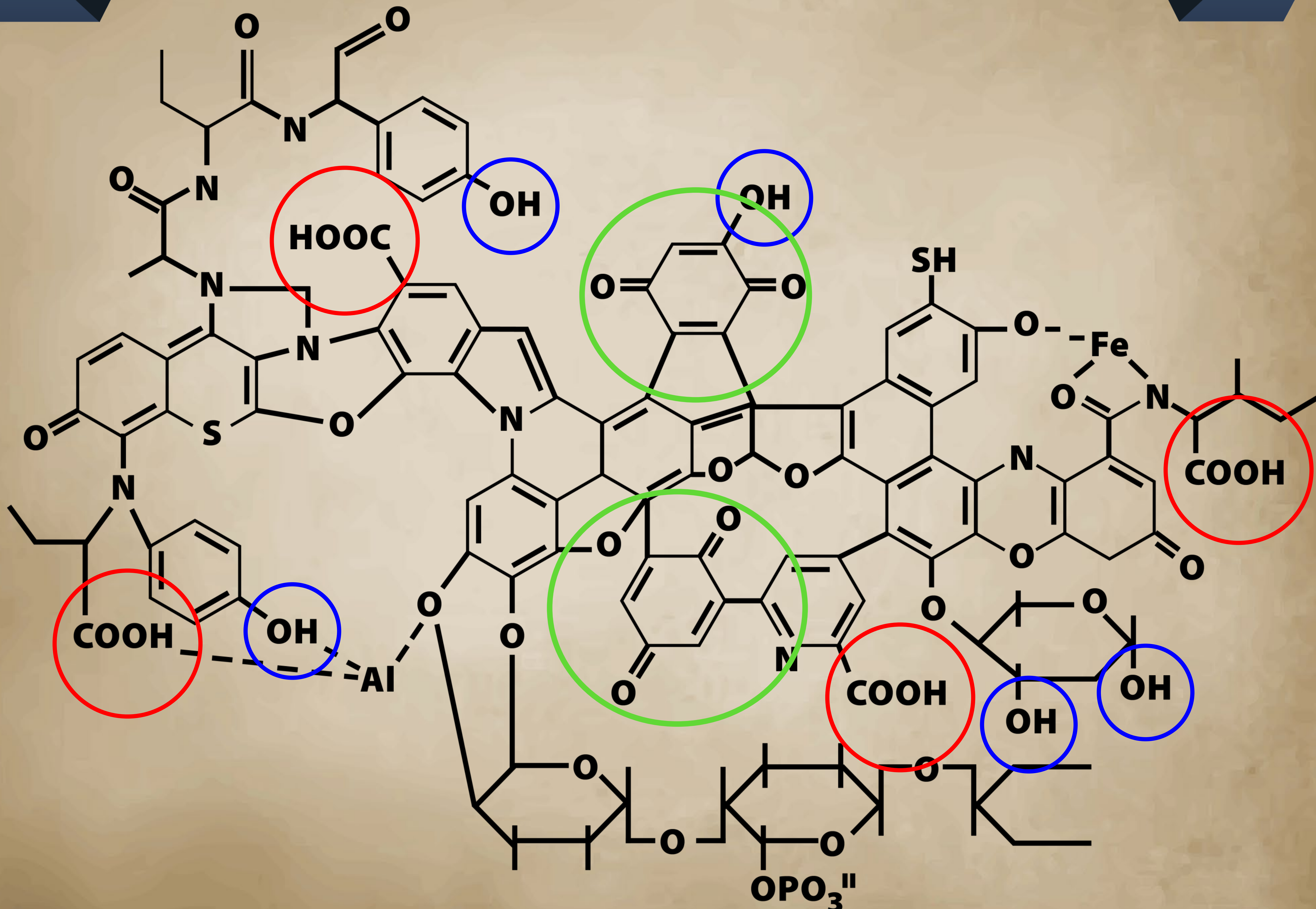
Several Important Concepts Regarding HS and Response to Stress in Plants

1. Humic Substance Functional Groups
2. Plasma Membrane
3. H^+-ATPase
4. Reactive Oxygen Species/Antioxidants
5. Initial Metabolic Events in Plant Stress Response
6. Effect of extracellular electron acceptors

Evidence that Humic Substances Elicit the same Metabolic Events as Plant Stressors

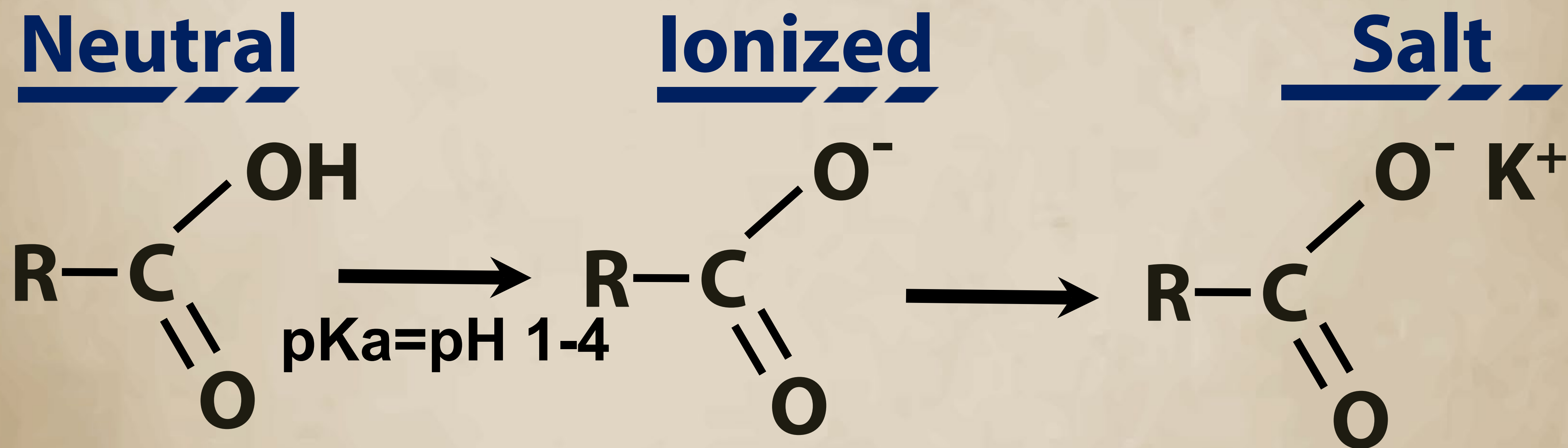
1. Presence of electron shuttles (quinones) in HS
2. PM depolarization by HA
3. Increase Ca^{2+} flux leading to increase $[Ca^{2+}]_{cyt}$
4. Increase in titer of Ca^{2+}-binding proteins
5. Increased H^+-ATPase activity

Conceptual Structure of Humic Acid



Primary Acidic Functional Groups

- Two most important groups are;
  - carboxylic acid groups (COOH)
  - Phenolic hydroxyl groups (C-OH)

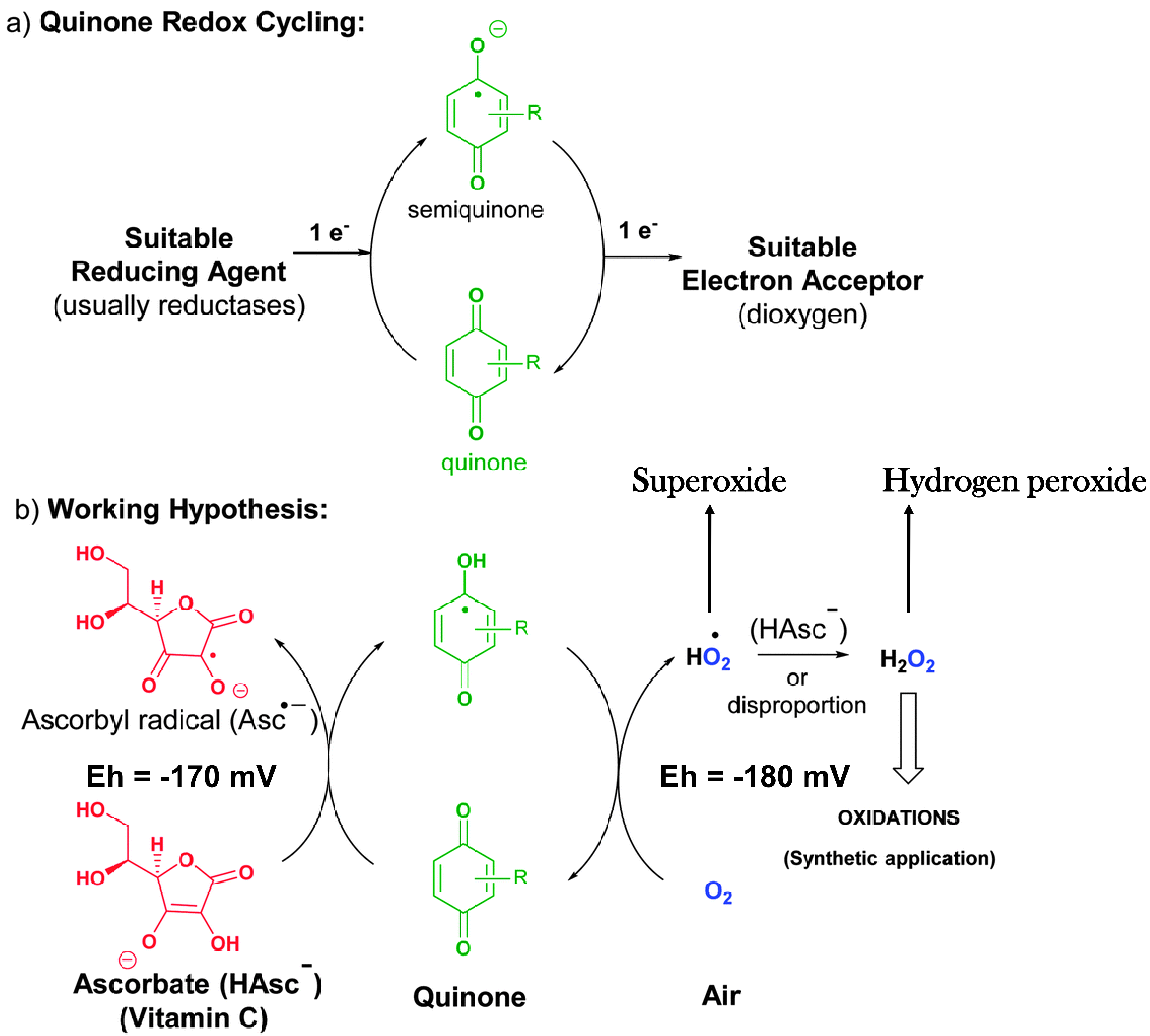


$R-C-OH \longrightarrow R-C-O^{-} \longrightarrow R-C-O^{-}K^{+}$

pKa=pH 7-9

pH-dependent CEC

Quinones

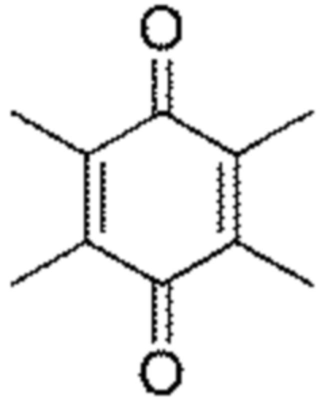


Quinones are redox active and can function as extracellular/intracellular electron acceptors

p-benzoquinone

$E^{o\prime}$=+78mV

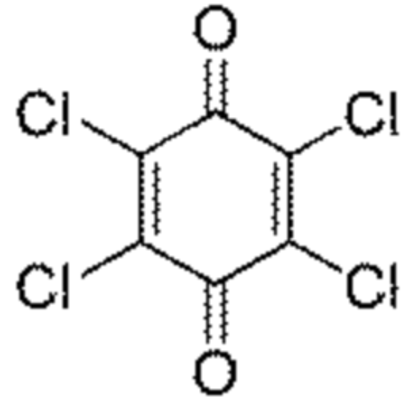
71%



duroquinone

$E^{o\prime}$=-260mV

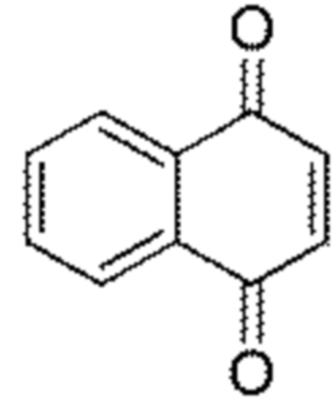
65%



chloranil

$E^{o\prime}$=+650mV

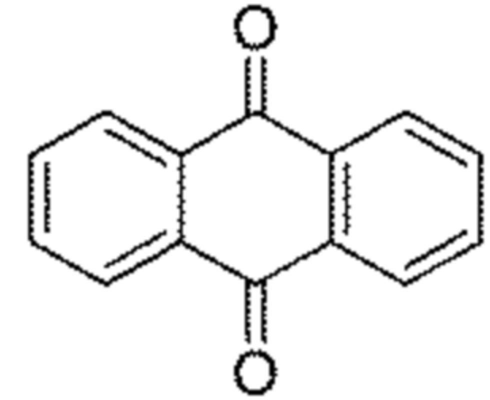
17%



naphtoquinone

$E^{o\prime}$=-140mV

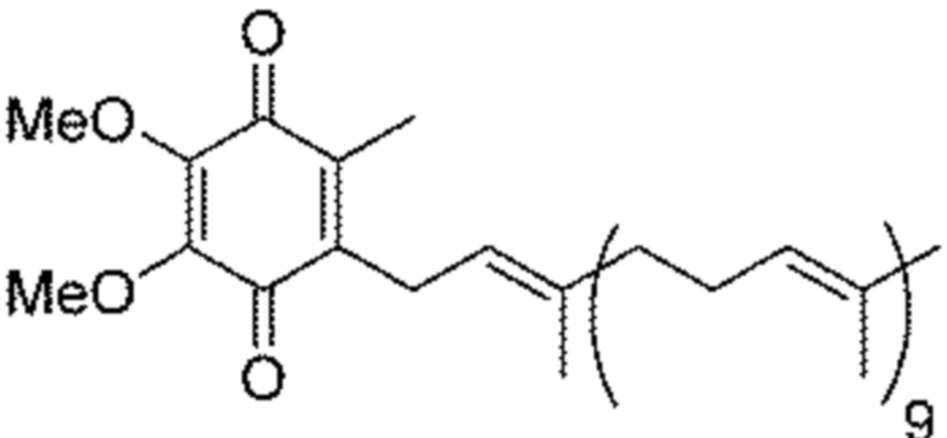
55%



antraquinone

$E^{o\prime}$=-445mV

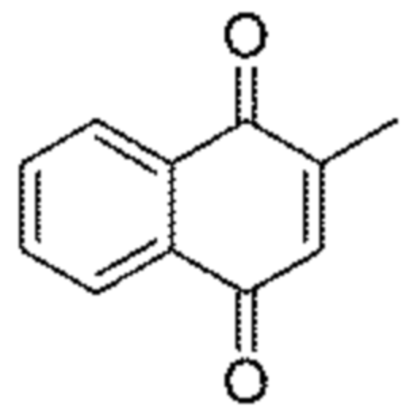
56%



Ubiquinone
(Coenzyme Q_{10})

$E^{o\prime}$=-230mV

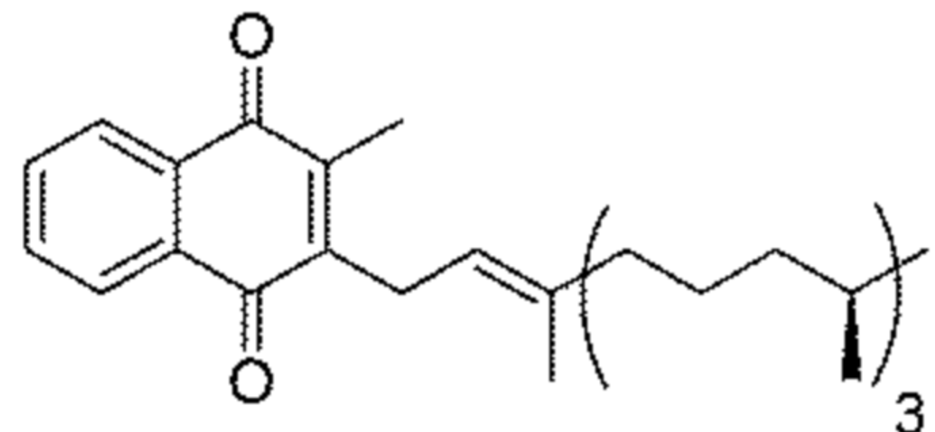
41%



Menadione
(vitamin K_3)

$E^{o\prime}$=-203mV

87%



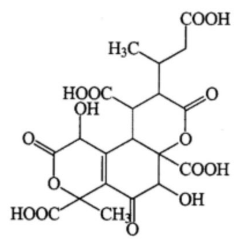
Phylloquinone
(vitamin K_1)

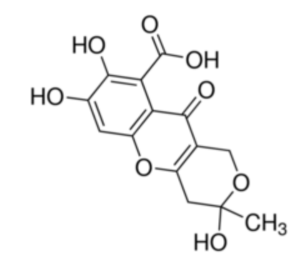
$E^{o\prime}$=-170mV

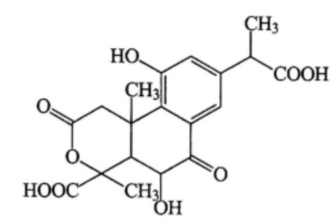
43%

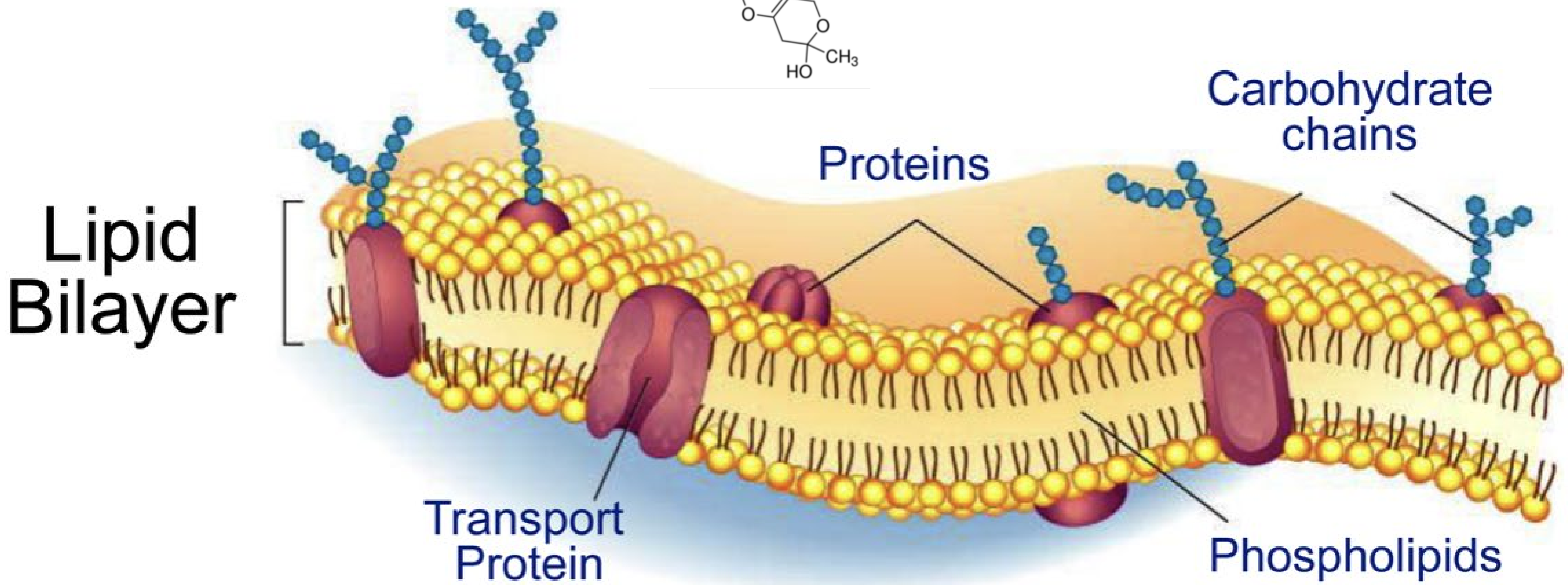
Plasma Membrane

Outside of cell (apoplast)



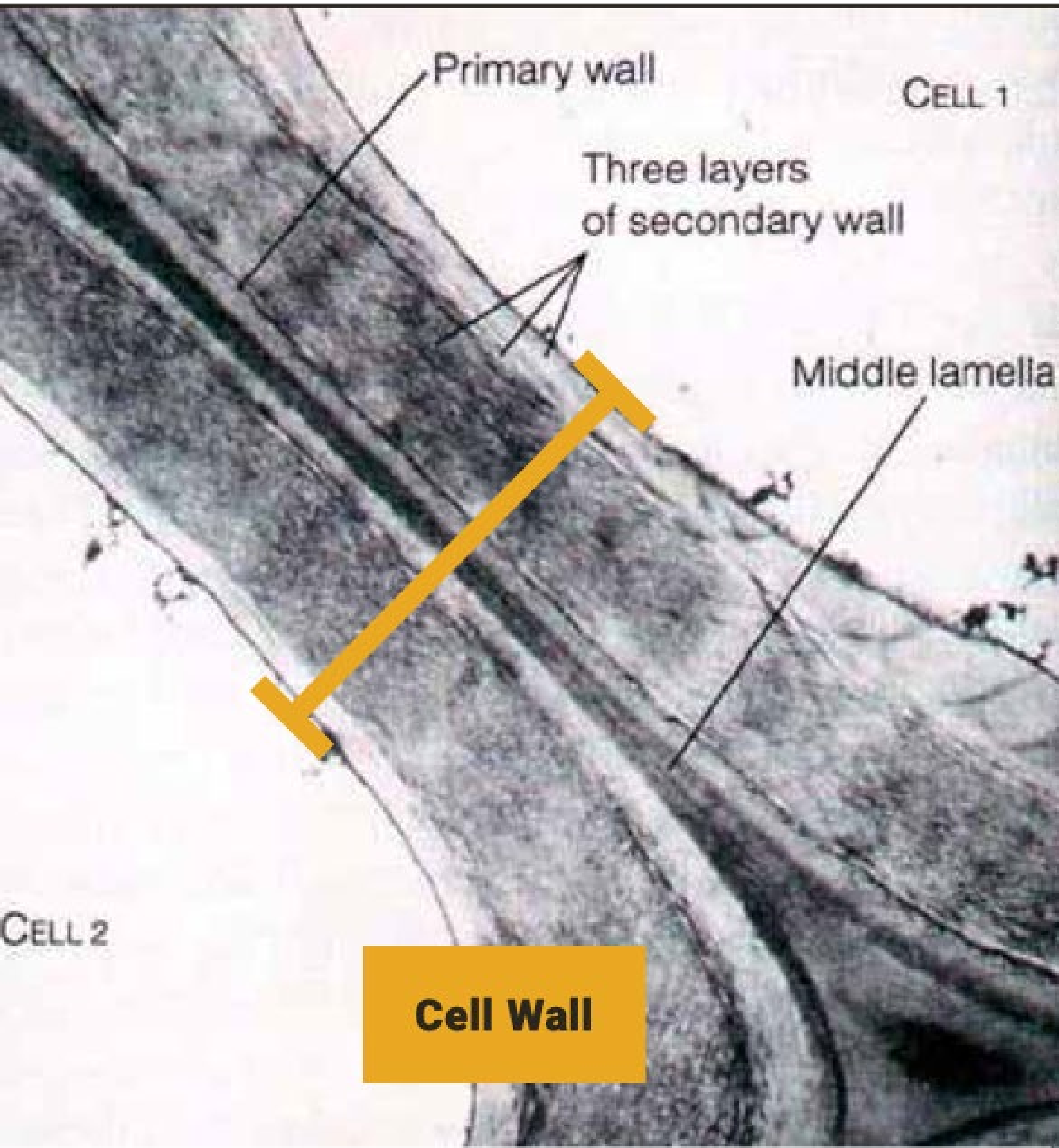


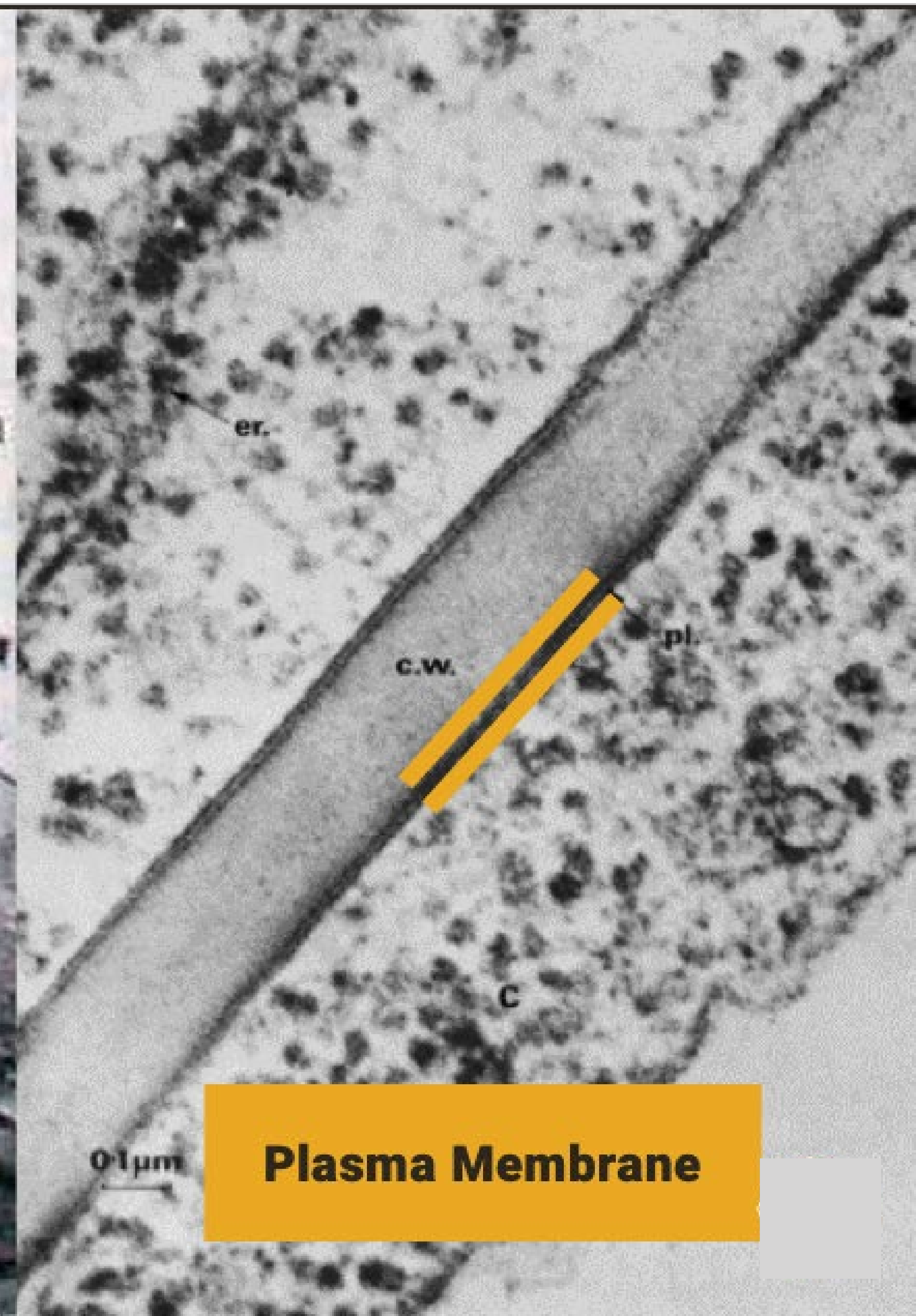




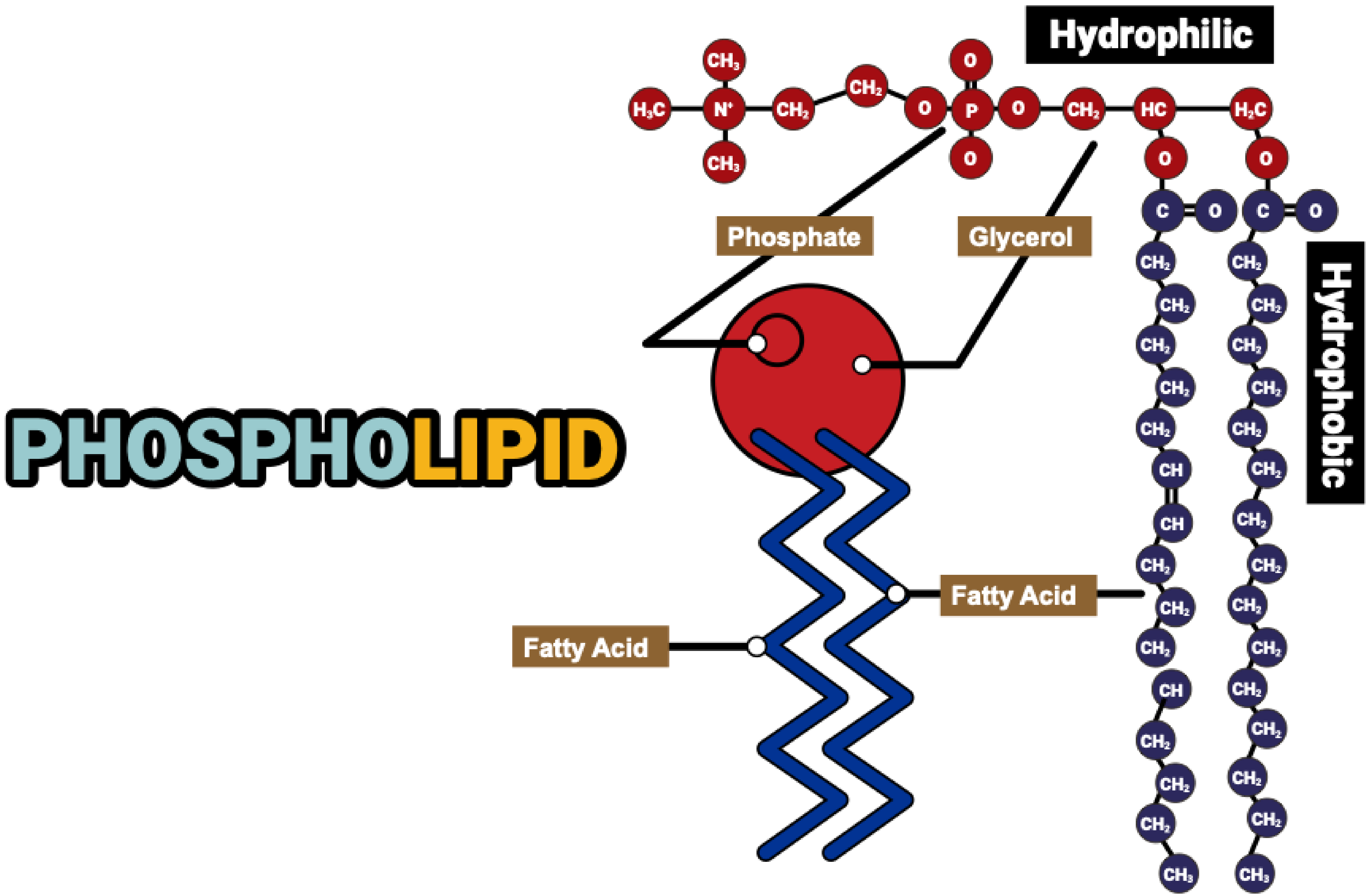
Inside of cell (cytoplasm)

Gatekeeper of the Cell

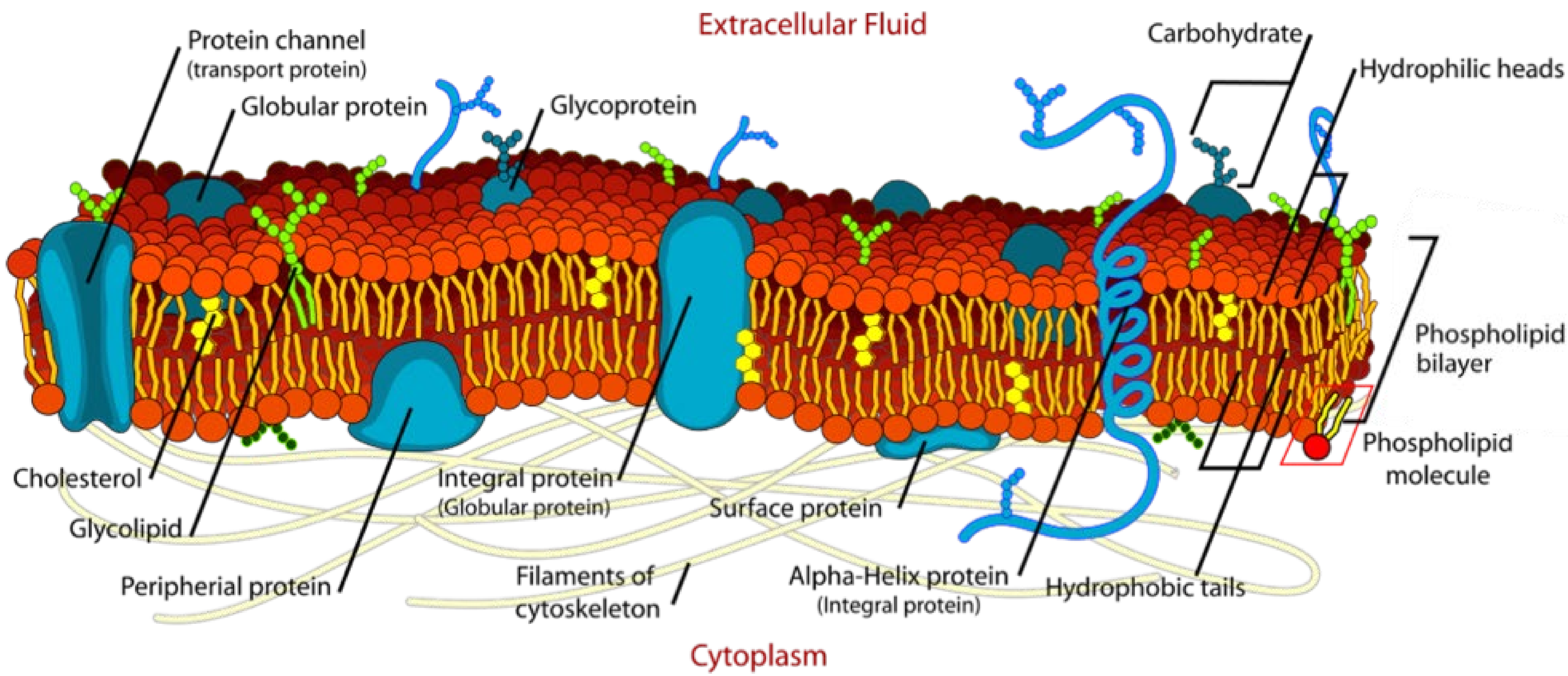




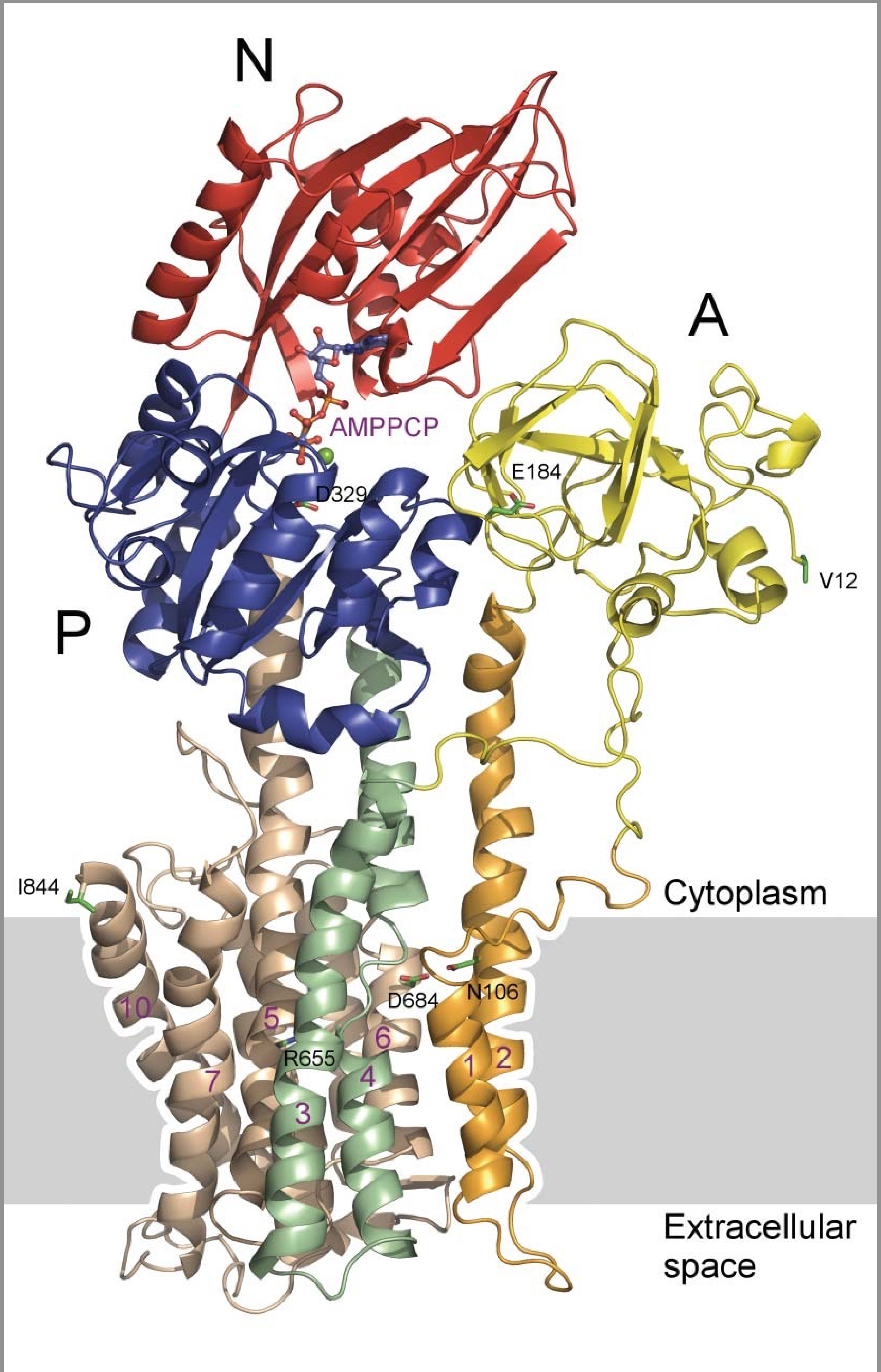
Plant Cell Membranes



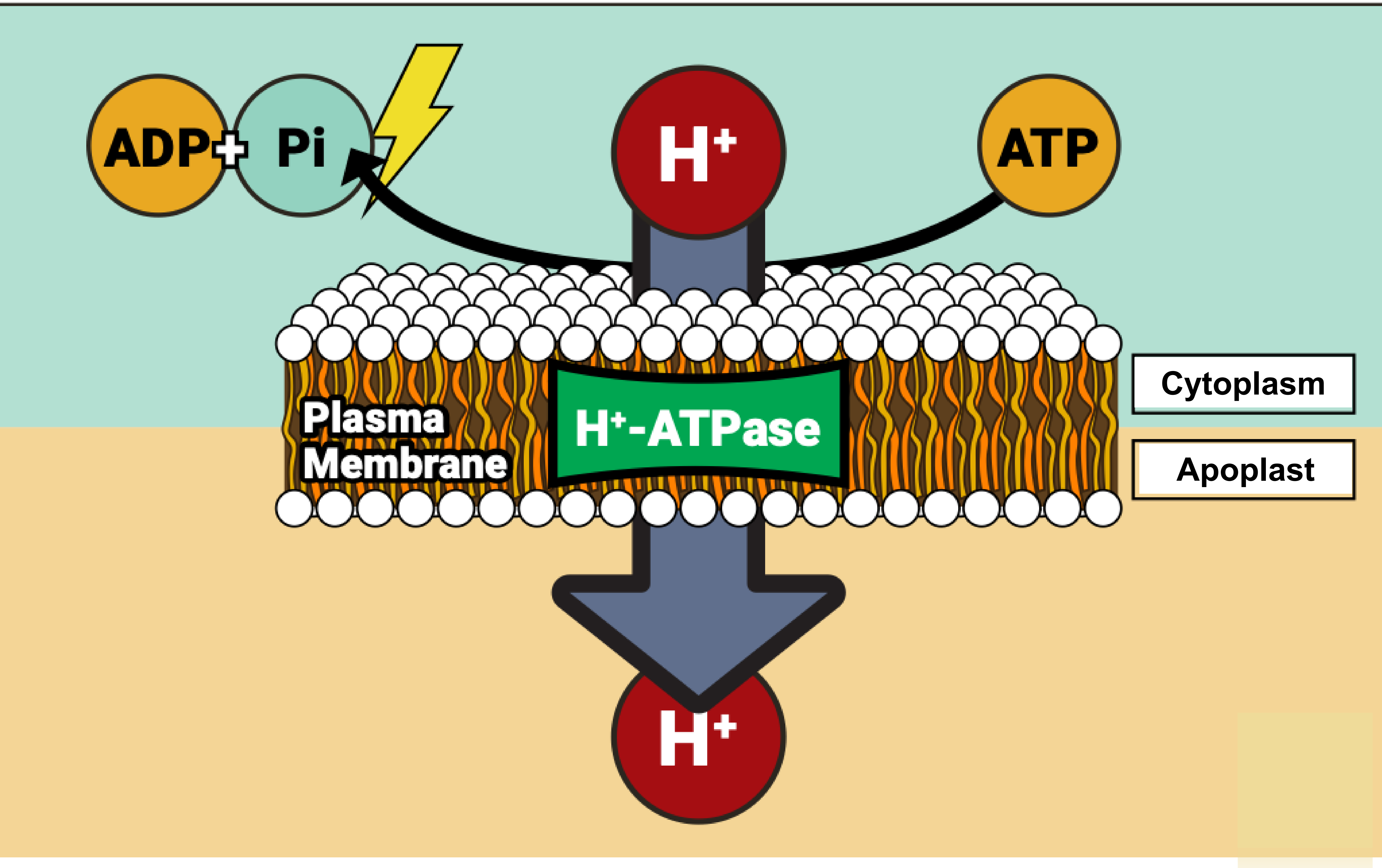
Plasma Membrane - X Section



Plasma Membrane H+-ATPase

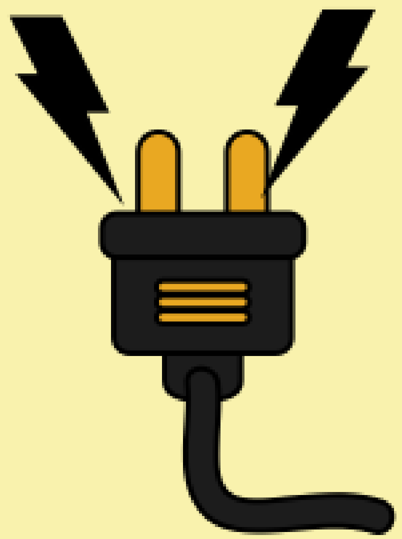


H^+-ATPases



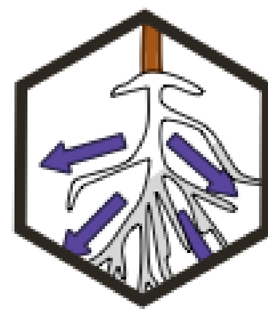
Functions of

Plasma Membrane H^+-ATPase

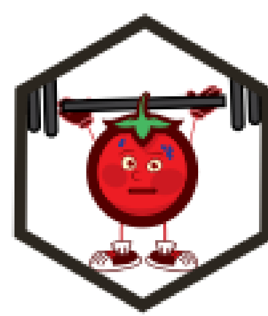


Generation of transmembrane electrochemical potential to **provide energy to drive** the flux of **nutrients** and solutes into and out of the cell

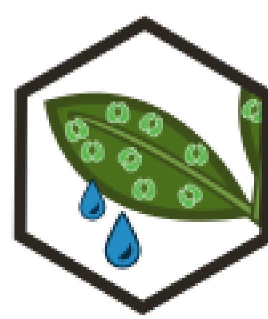
Maintenance of cytoplasmic pH **(pH 7.0 – 7.5)**



Plays a major role in **root elongation**



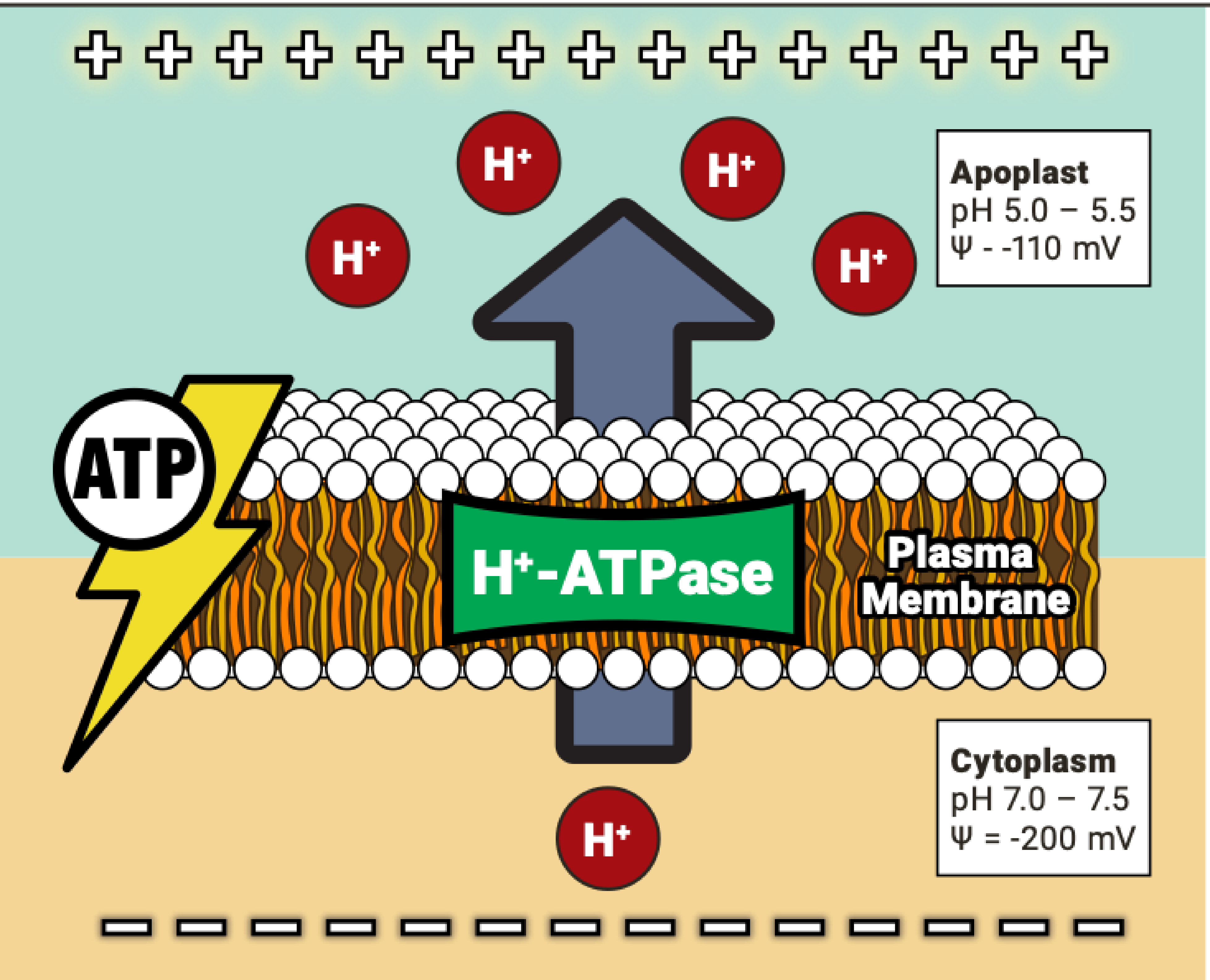
Role in **stress response**



Involved in **stomatal opening** and **closing**

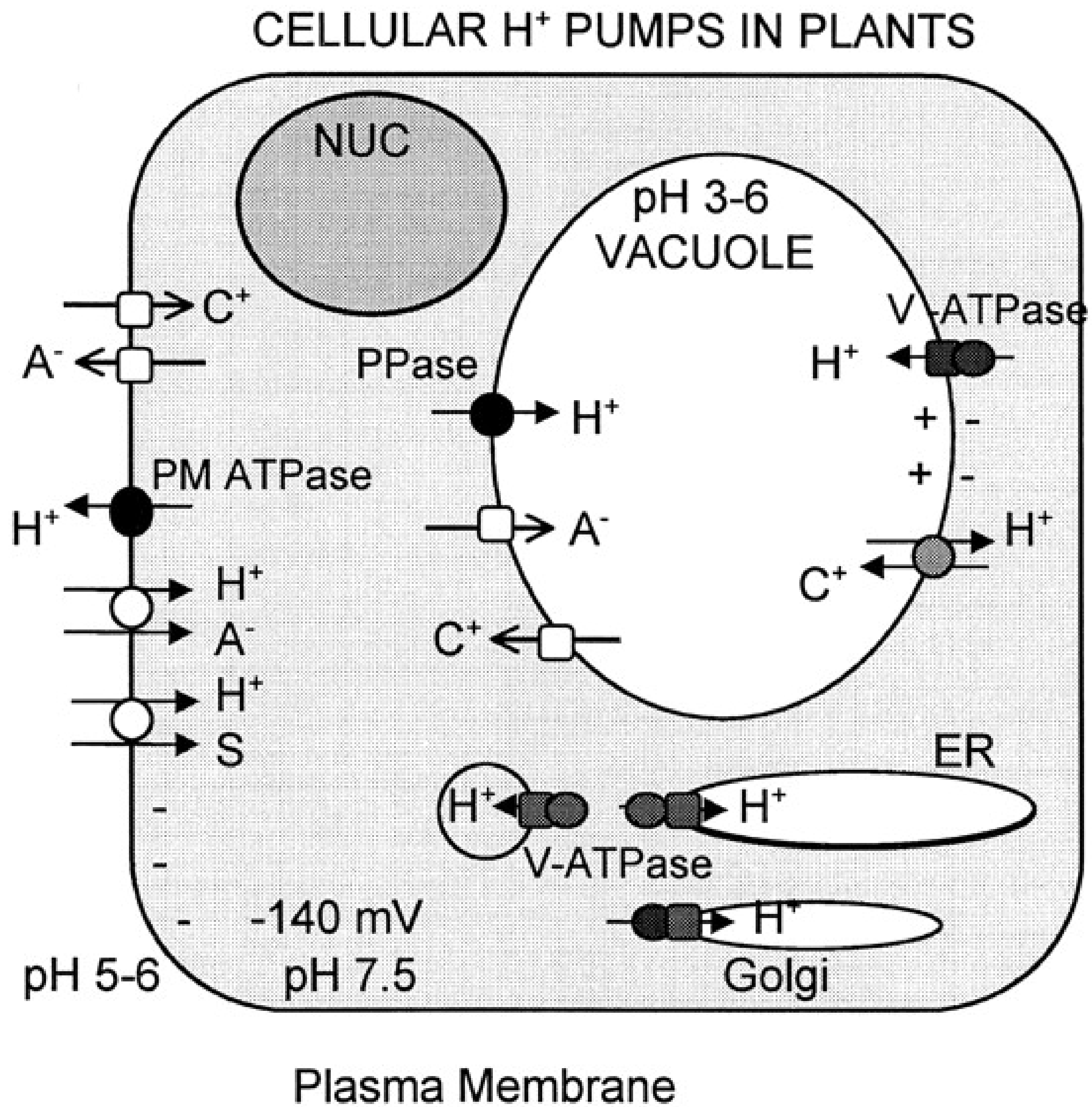
H^+-ATPases

Proton Motive Force **(PMF)**



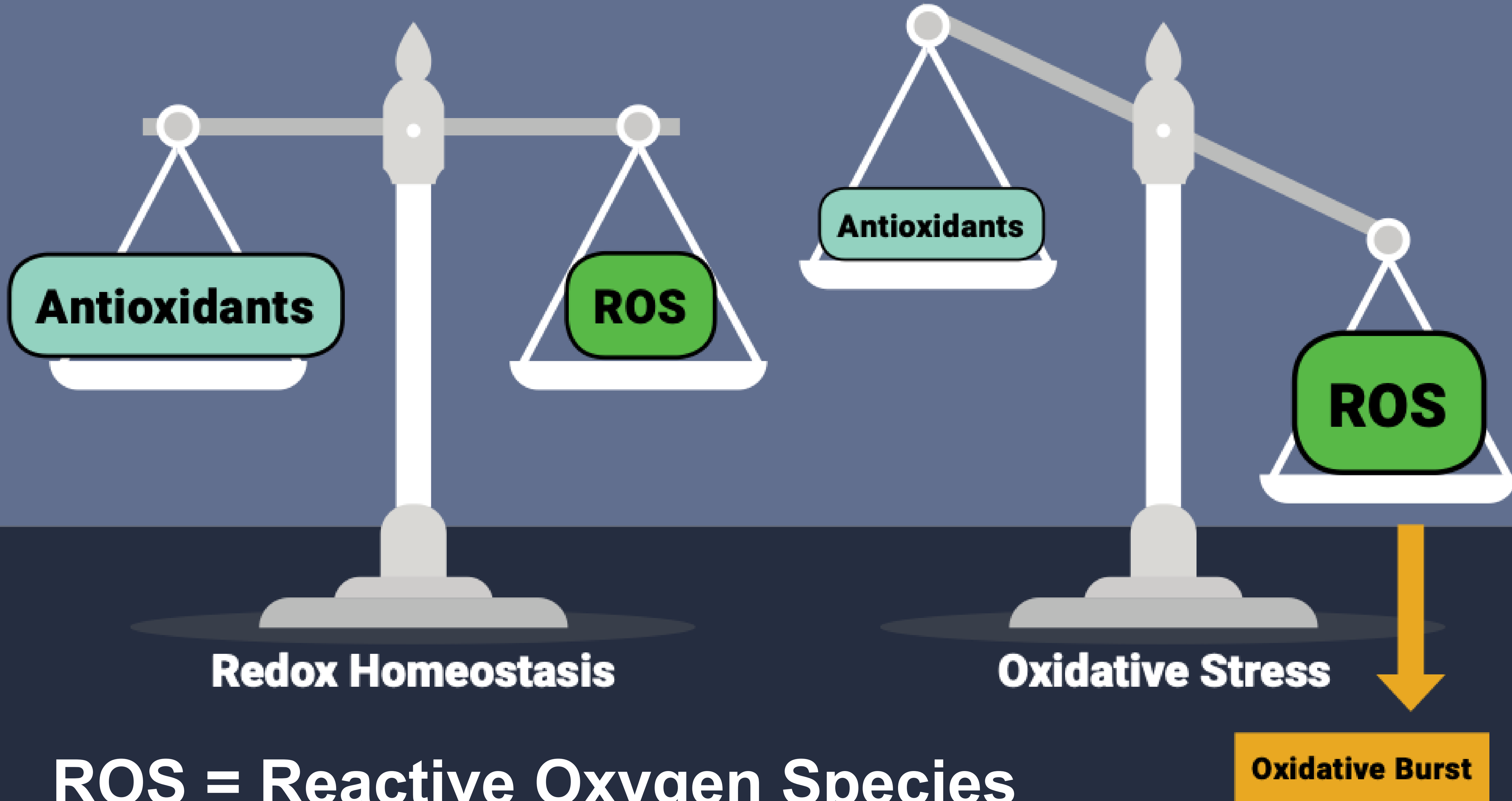
Plasma membrane **PMF** composed of a electrical gradient (Δψ) and a proton chemical concentration gradient (ΔpH) = electrochemical potential gradient

Three Types of H+ Pumps in Plant Cells. PM H+ ATPase extrudes H+ outside the cell generating a proton electrochemical gradient (-120 to -160 mV relative to the outside).



Heven Sze et al. Plant Cell 1999;11:677-689

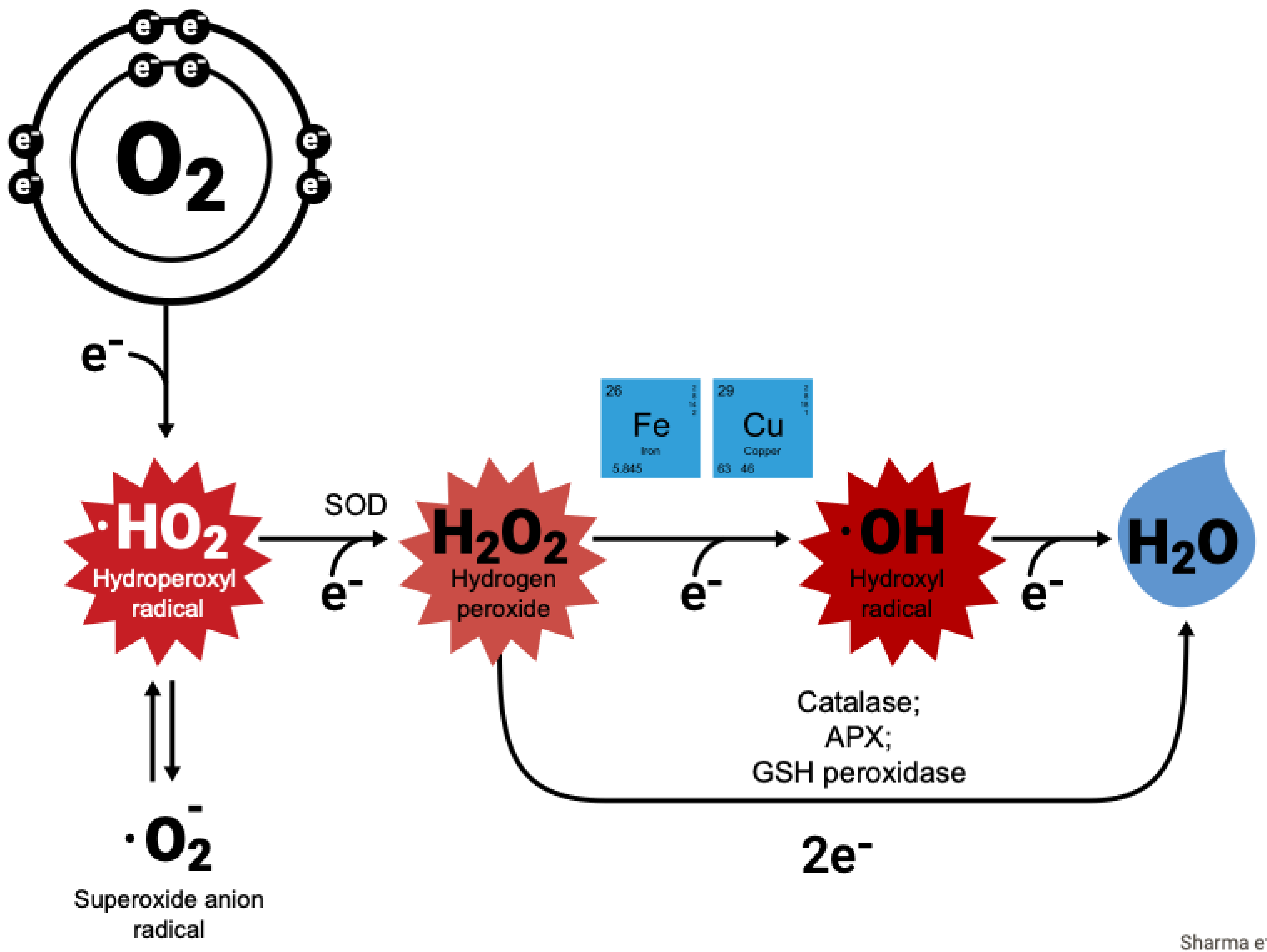
Redox Homeostasis



ROS = Reactive Oxygen Species

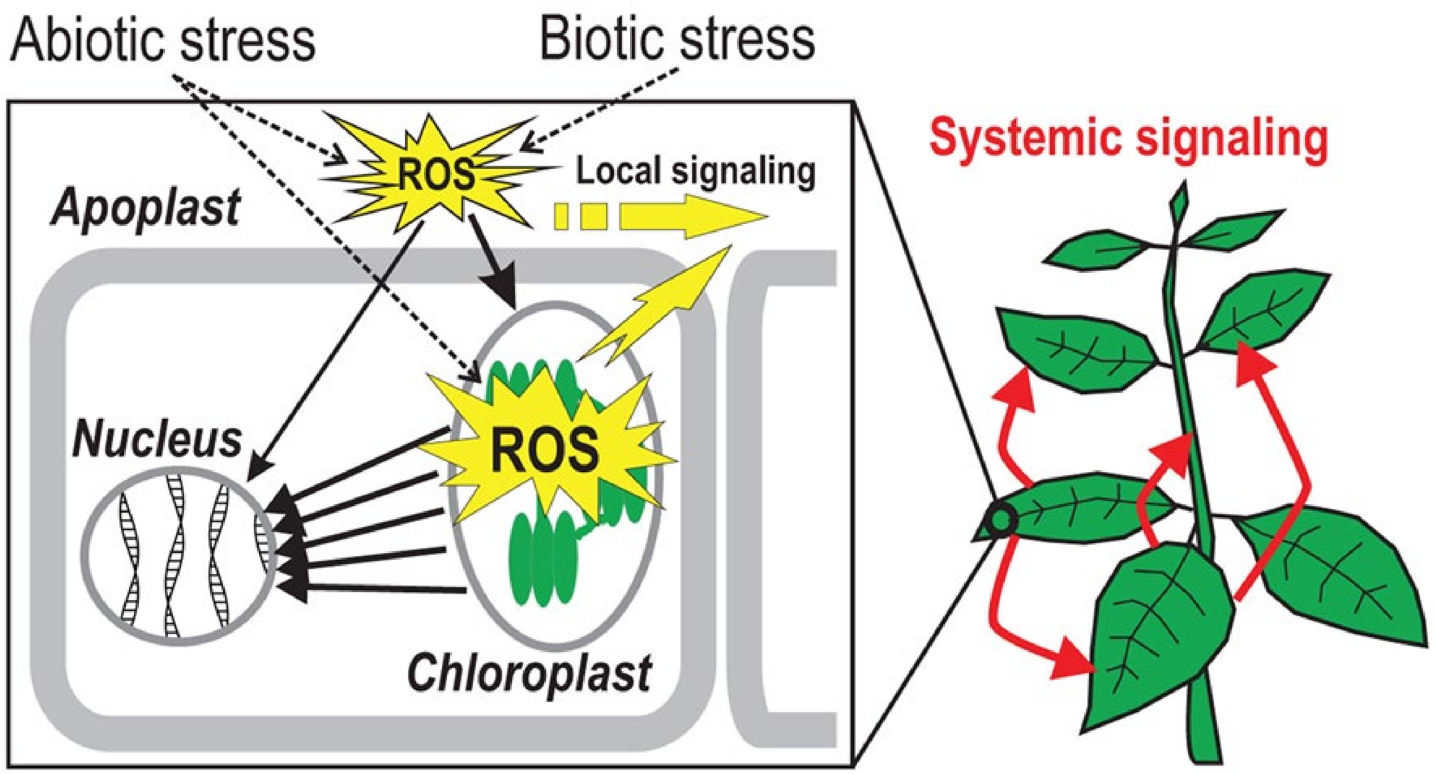
(from Zhang Y, Martin SG, Redox Proteins and Radiotherapy, Clinical Oncology (2014)

Reactive Oxygen Species (ROS)

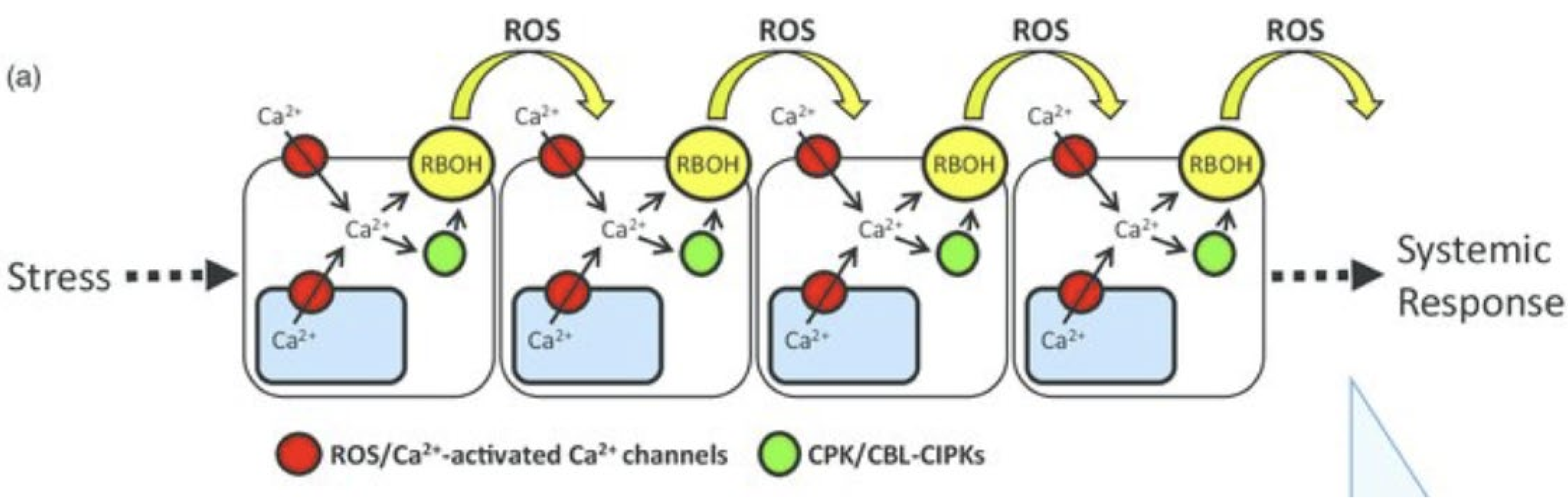


Sharma et al., 2012

ROS Talk



Abiotic stress
Biotic stress
ROS
Local signaling
Apoplast
Nucleus
ROS
Chloroplast
Systemic signaling



(a)
ROS
ROS
ROS
ROS
Ca2+
RBOH
Stress
Systemic Response
ROS/Ca2+-activated Ca2+ channels
CPK/CBL-CIPKs

Different ROS Play Different Roles

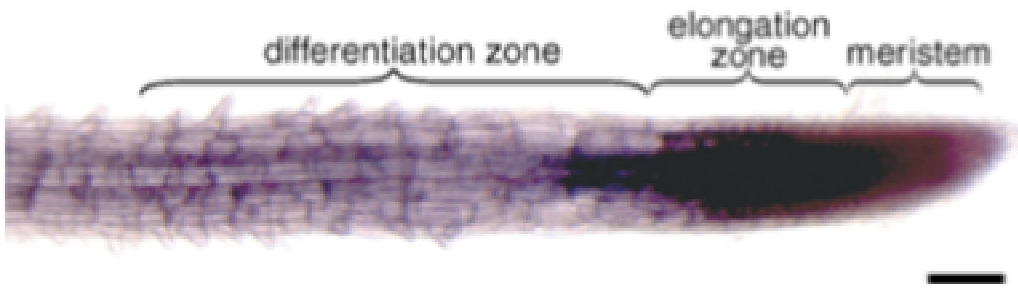
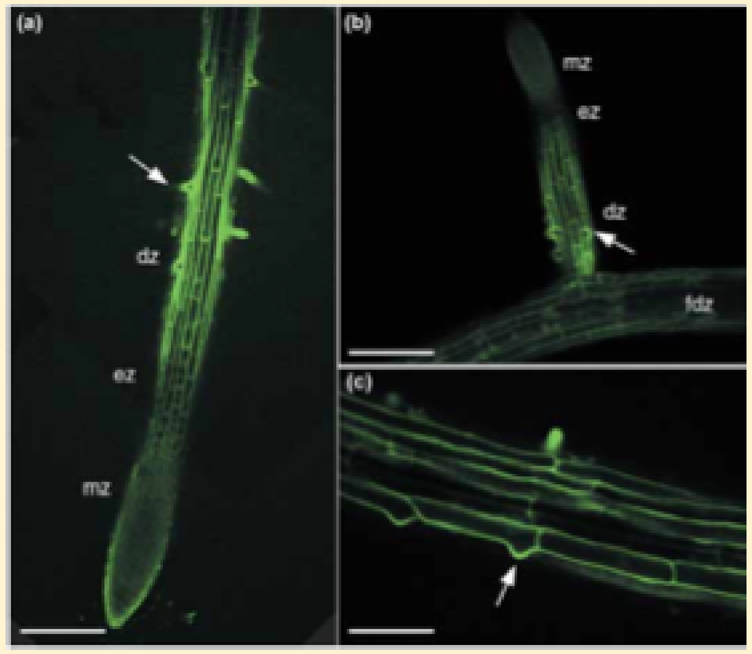


Fig. 4 Distribution of $O_2^{\bullet -}$ visualized by nitroblue tetrazolium (NBT) staining in *Arabidopsis* root tip. Bar, 100 μm.

Superoxide (O_2^-) accumulated in apoplast of elongation zone cells.

H_2O_2 found in differentiation zone and cell walls of root hairs in formation.



Dunand et al., 2007. Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their infouences on root development: possible interactions with peroxidases.New Phytologist 174: 332 – 341.

Antioxidant Defense System

Non Enzymatic Antioxidant Molecules	Primary ROS
Ascorbate (Vitamin C)	H_20_2, 0_2^-
Glutathione reduced (GSH)	H_20_2
β-Carotene	$^10_2$
α-tocopherol (Vitamin E)	ROOH, $^10_2$
Zeaxanthin	$^10_2$

Antioxidant Enzymes	Primary ROS
Superoxide dismutase (SOD)	O_2^-
Ascorbate peroxidase (APX)	H_20_2
Catalase (CAT)	H_20_2
Peroxidase (non-specific)	H_20_2
Glutathione peroxidase (GPX)	H_20_2, ROOH
Glutathione reductase (GR)	ROOH

Front Line Enzymatic Antioxidants

SOD and CAT

SOD

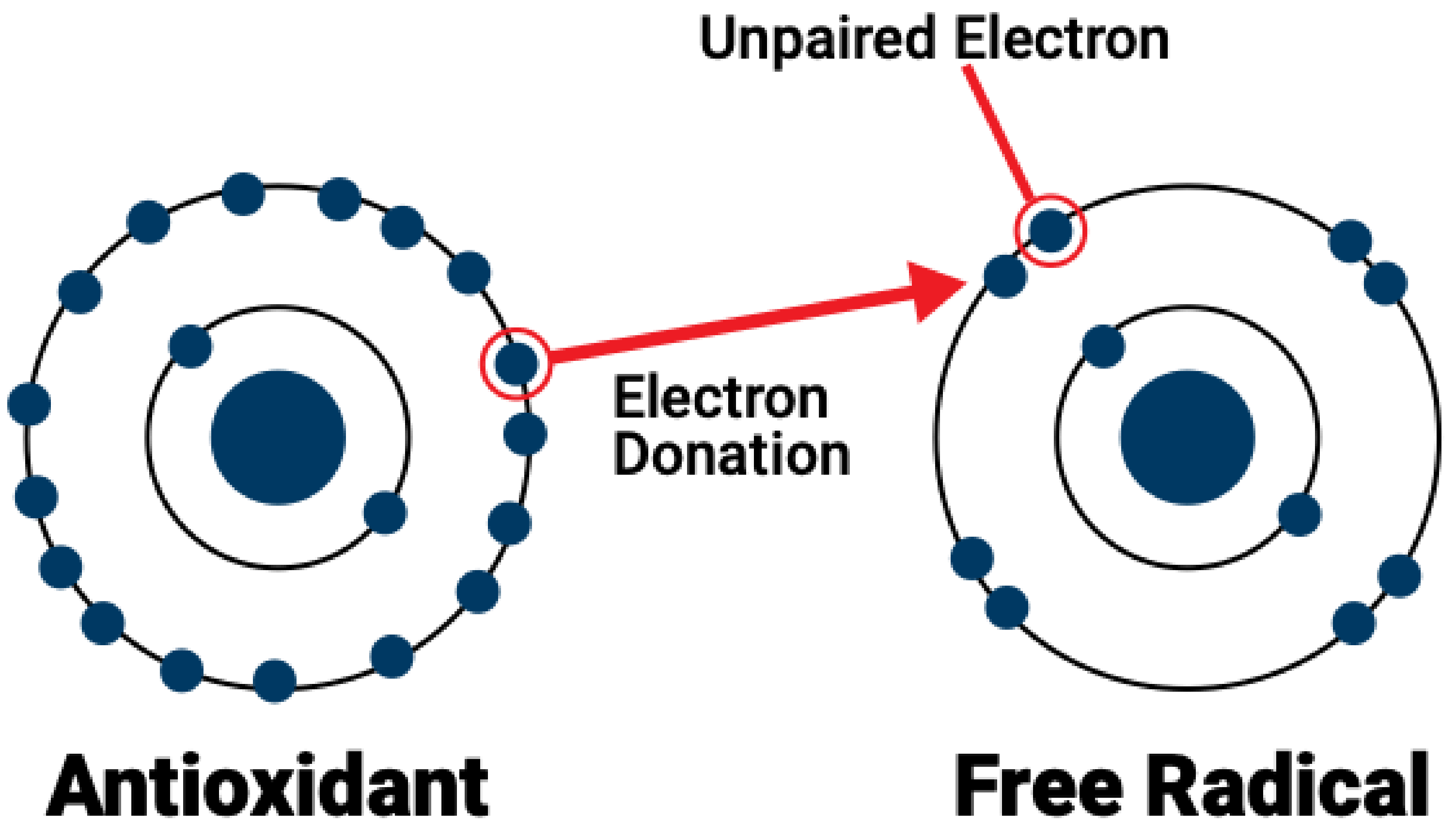
$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \; (K_2 = 2.4 \times 10^9 \; M^{-1} \; s^{-1})$$

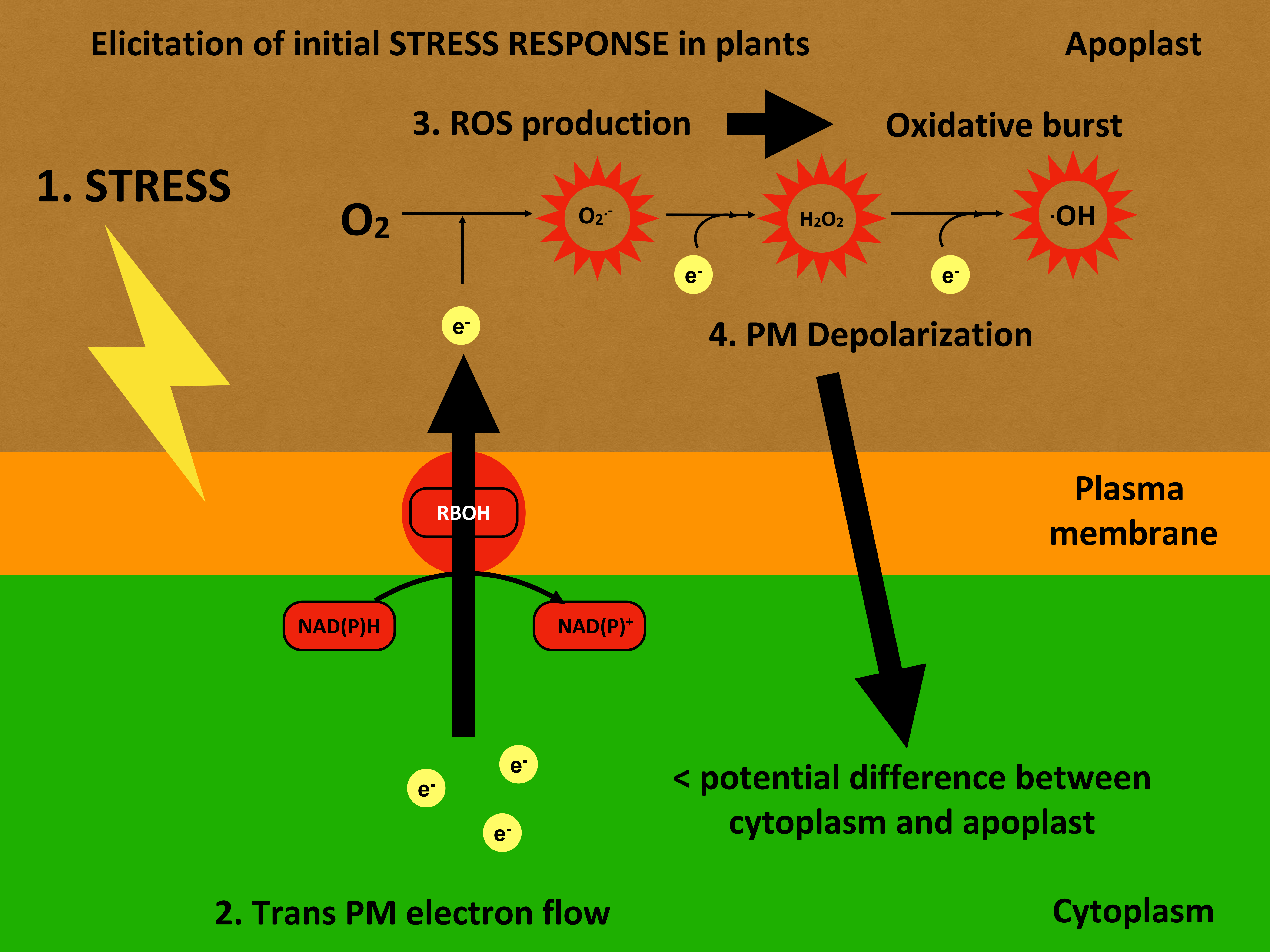
CAT

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \; (K_1 = 1.7 \times 10^7 \; M^{-1} \; s^{-1})$$

PX

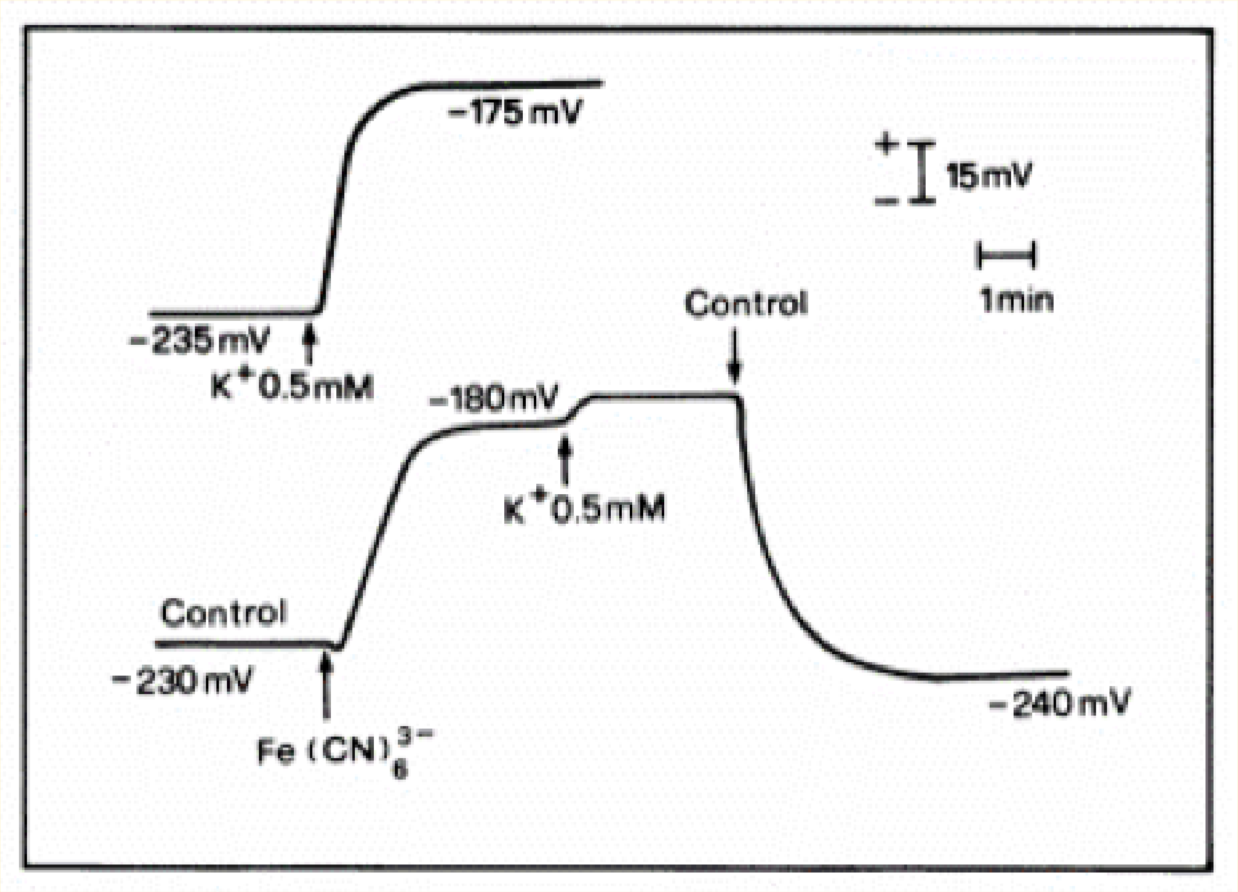
$$H_2O_2 + R(OH)_2 \rightarrow 2H_2O + R(O)_2 \; (K_4 = 0.2\text{–}1 \times 10^3 \; M^{-1} \; s^{-1})$$





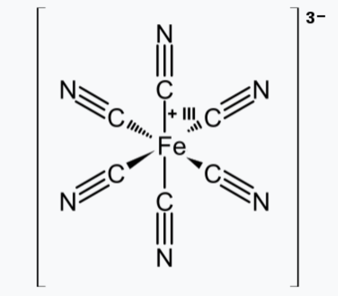
Elicitation of initial STRESS RESPONSE in plants
Apoplast
3. ROS production
Oxidative burst
1. STRESS
O_2
$O_2^{\cdot -}$
H_2O_2
·OH
e-
e-
e-
4. PM Depolarization
Plasma membrane
RBOH
NAD(P)H
NAD(P)+
e-
e-
e-
< potential difference between cytoplasm and apoplast
2. Trans PM electron flow
Cytoplasm

Membrane depolarization- Ferricyanide

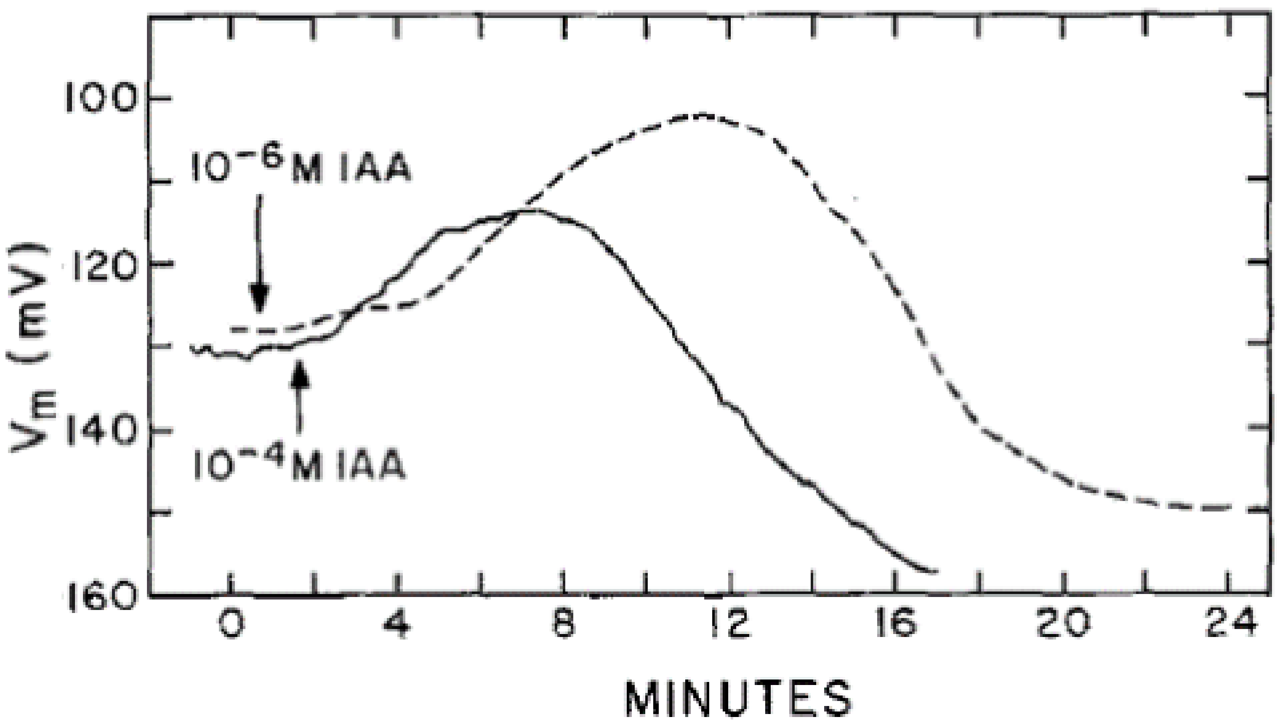


Marre' et al., 1988. Plasmalemma redox activity and H+ extrusion. Plant Physiol. 87: 25 – 29.

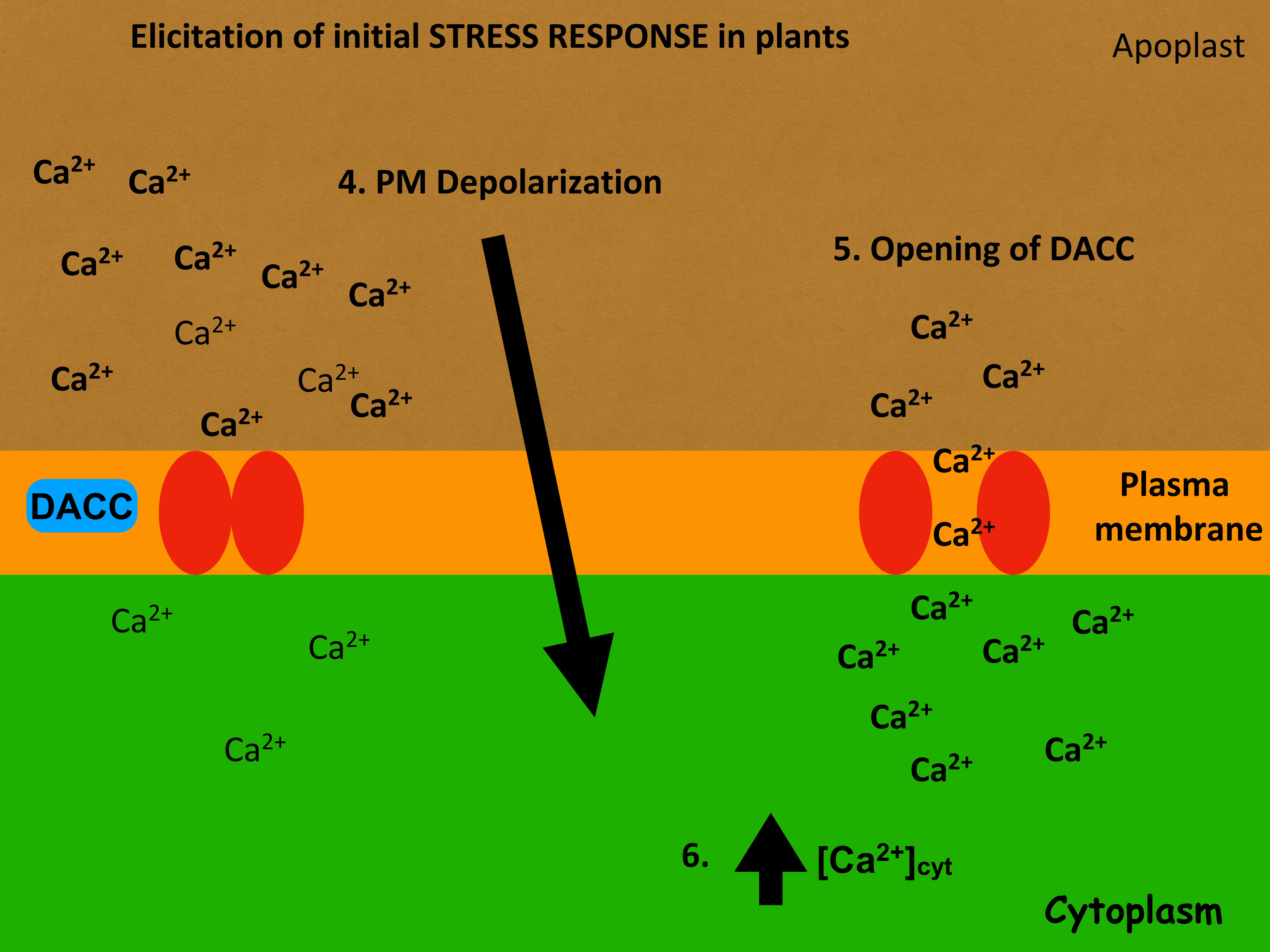
Ferricyanide



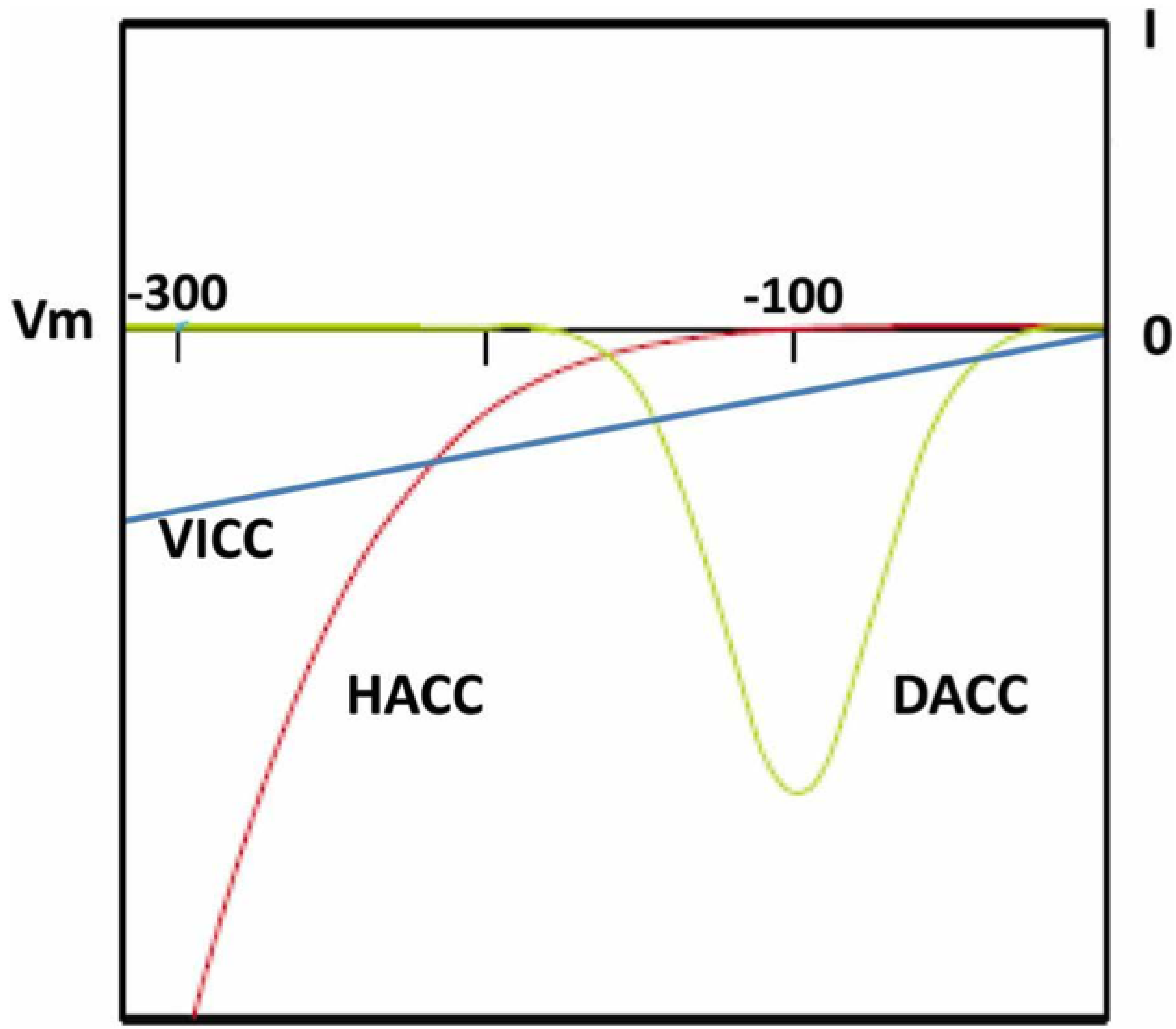
Membrane depolarization- IAA



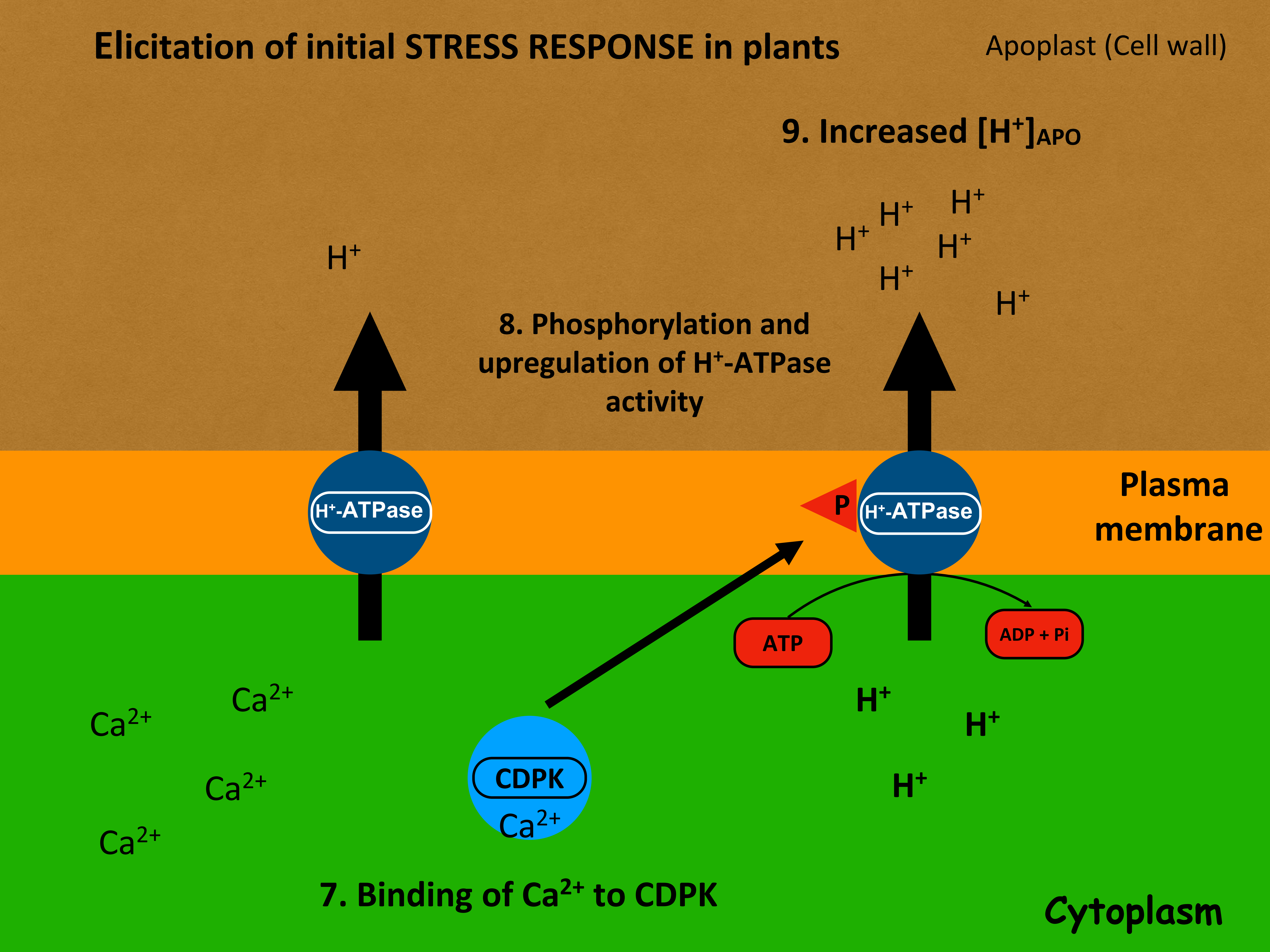
Bates and Goldsmith, 1983. Rapid response of the plasma-Membrane potential in oat coleoptiles to auxin and other Weak acids. Planta 159: 231 – 237.


Elicitation of initial STRESS RESPONSE in plants
Apoplast
4. PM Depolarization
5. Opening of DACC
DACC
Plasma membrane
6. [Ca2+]cyt
Cytoplasm
Ca2+

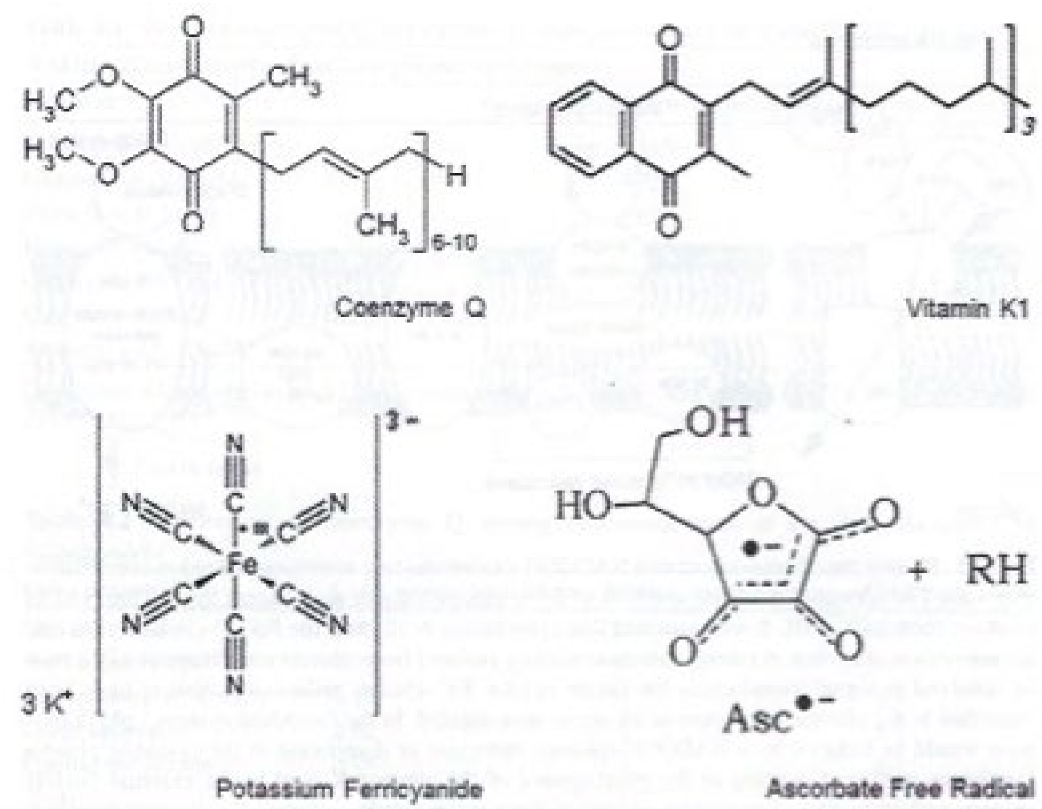
Co-residence of different **Ca^{2+}** channels in the plasma membrane affords **variable Ca^{2+} influx from the apoplast,** controlled by membrane voltage.



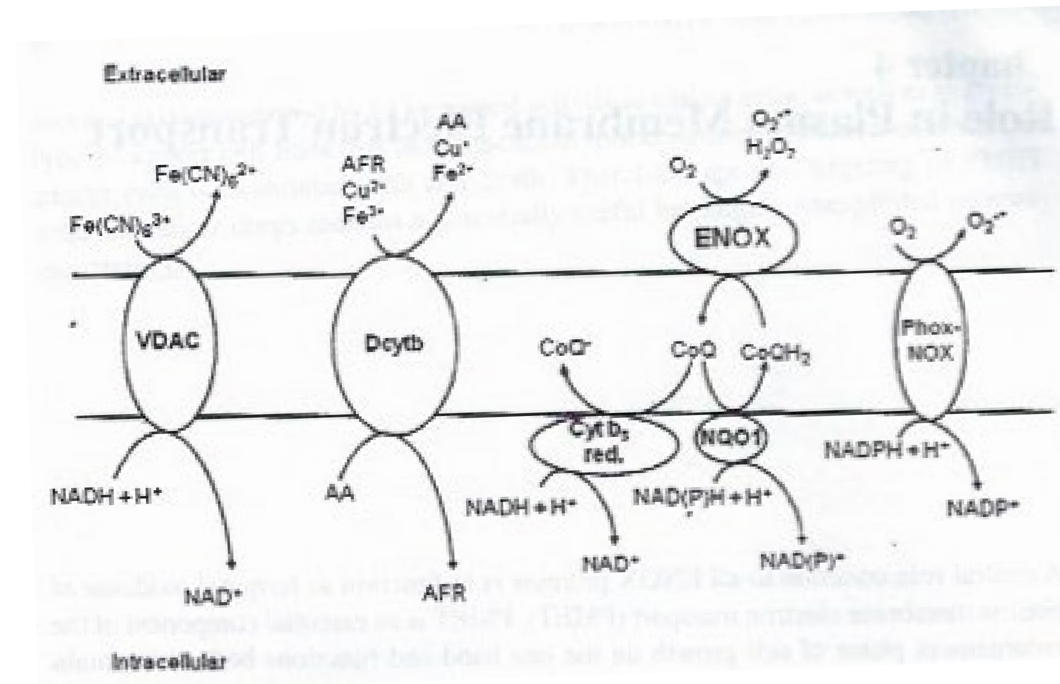
Stéphanie M. Swarbreck et al. Plant Physiol. 2013;163: 514-522. ©2013 by American Society of Plant Biologists


Elicitation of initial STRESS RESPONSE in plants
Apoplast (Cell wall)
9. Increased $[H^+]_{APO}$
H^+
H^+
H^+
H^+
H^+
H^+
H^+
8. Phosphorylation and upregulation of H^+-ATPase activity
H^+-ATPase
P
H^+-ATPase
Plasma membrane
ATP
ADP + Pi
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
CDPK
Ca^{2+}
H^+
H^+
H^+
7. Binding of Ca^{2+} to CDPK
Cytoplasm

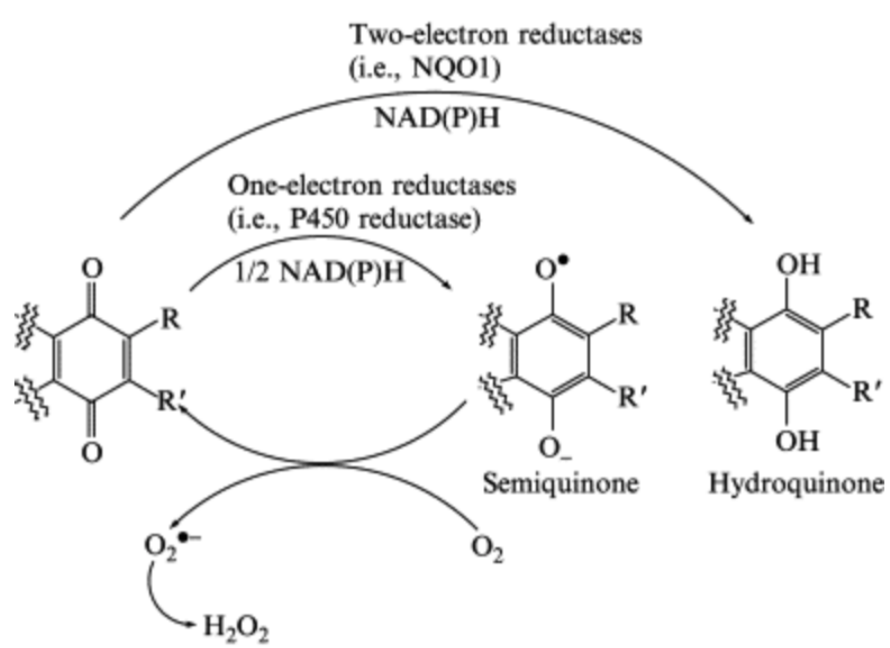
Extracellular Electron Acceptors



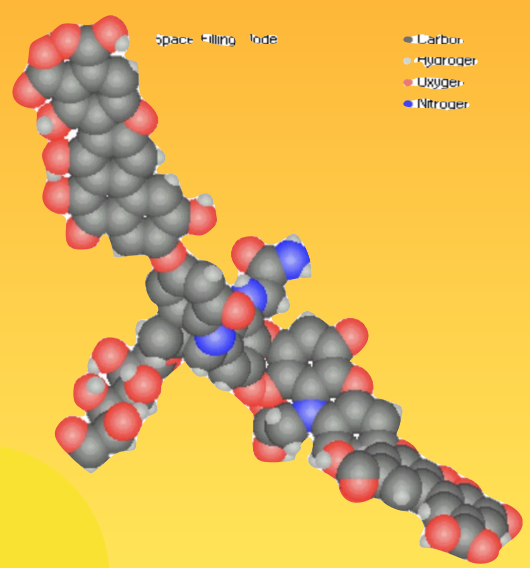
Transmembrane Electron Transport



Quinones can undergo either 1- or 2- electron reductions



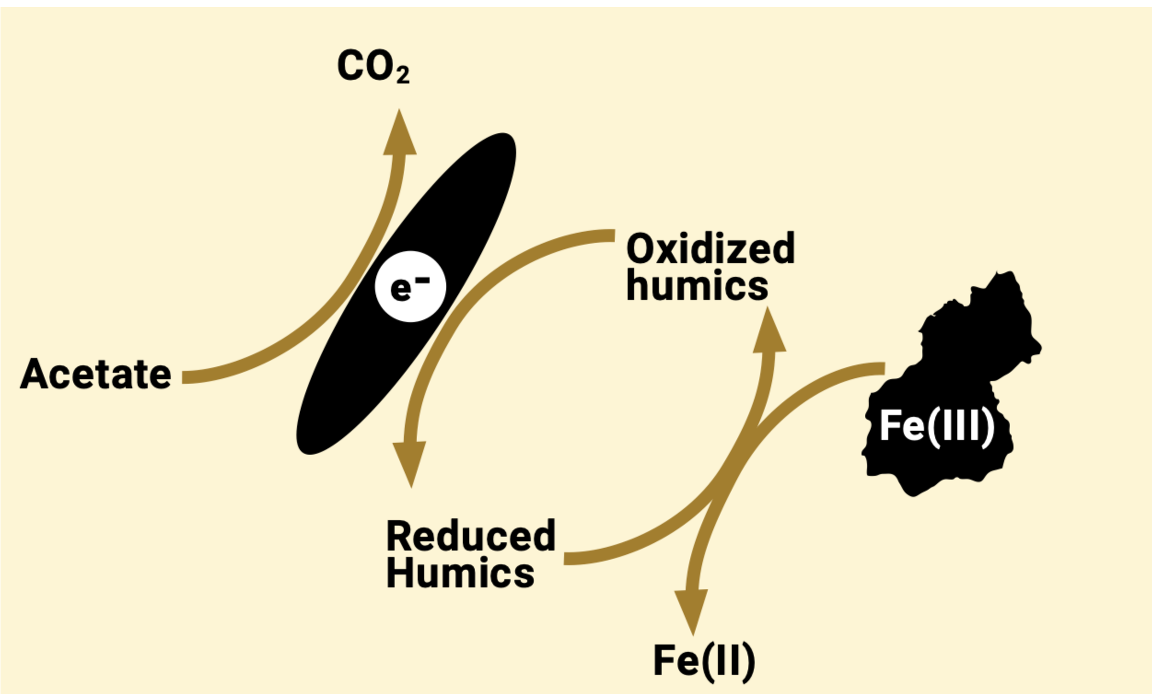
Evidence That Humic Substances Elicit the Same Metabolic Events as Plant Stressors



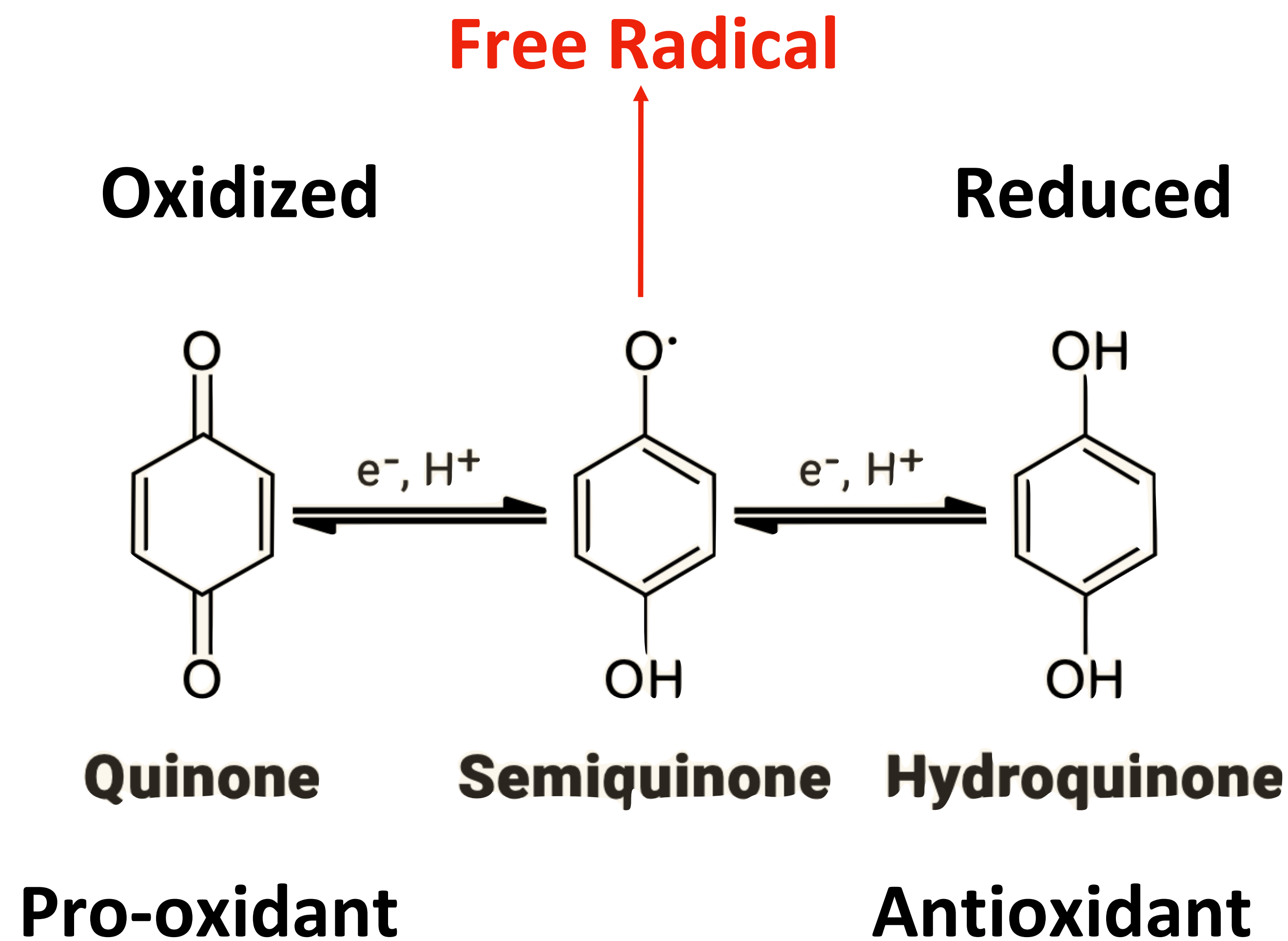
- > PM electron flow
- > ROS + antioxidant Production
- PM depolarization
- > $[Ca^{2+}]_{cyt}$
- > H+-ATPase activity

Result = Priming effect to enable plant to better withstand stress

Evidence that HS can act as electron acceptors



Lovley et al., 1996. Humic substances as electron acceptors for microbial respiration. Nature 382: 445 – 448.



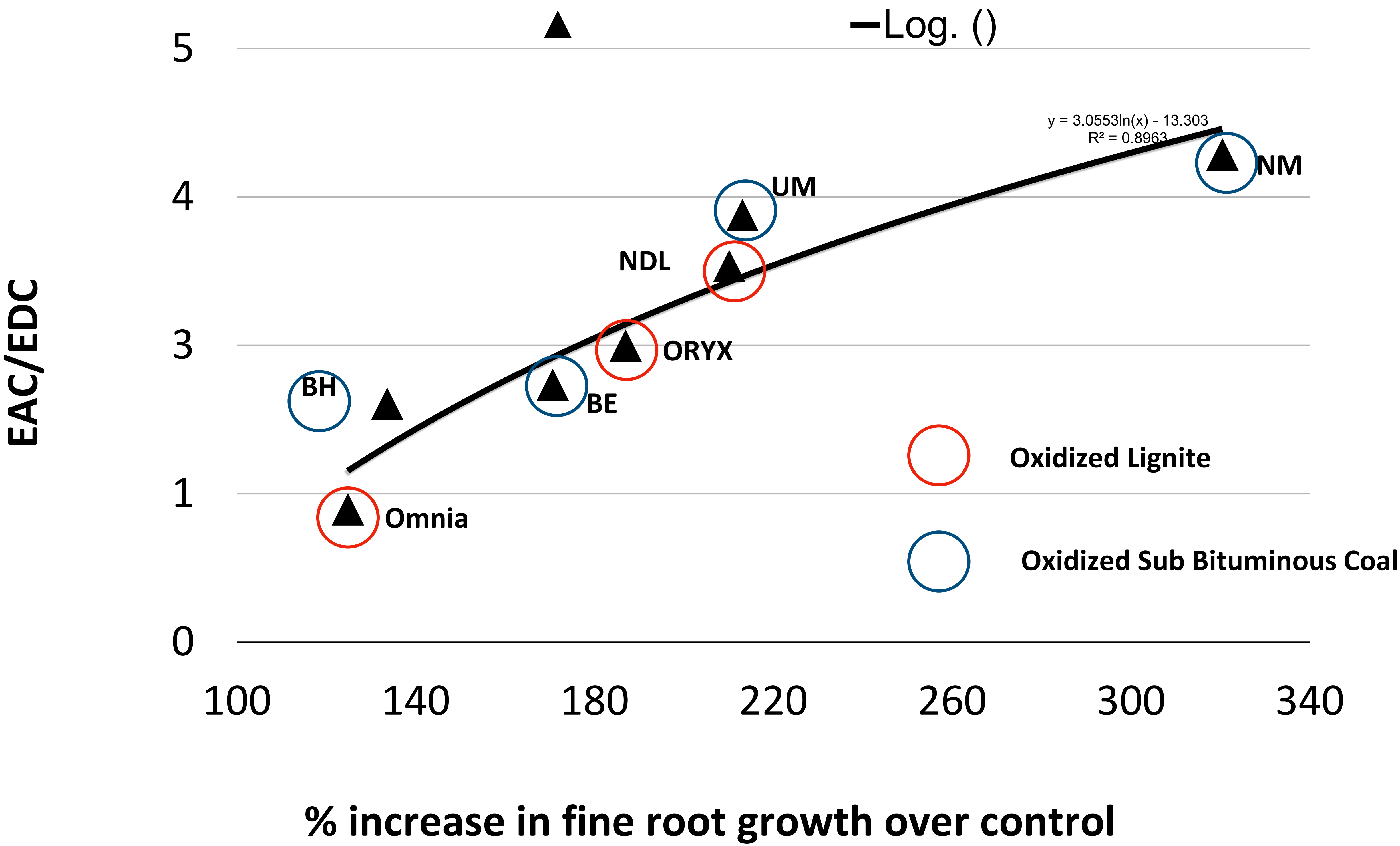
Primary source of electron transfer in HS is QUINONES

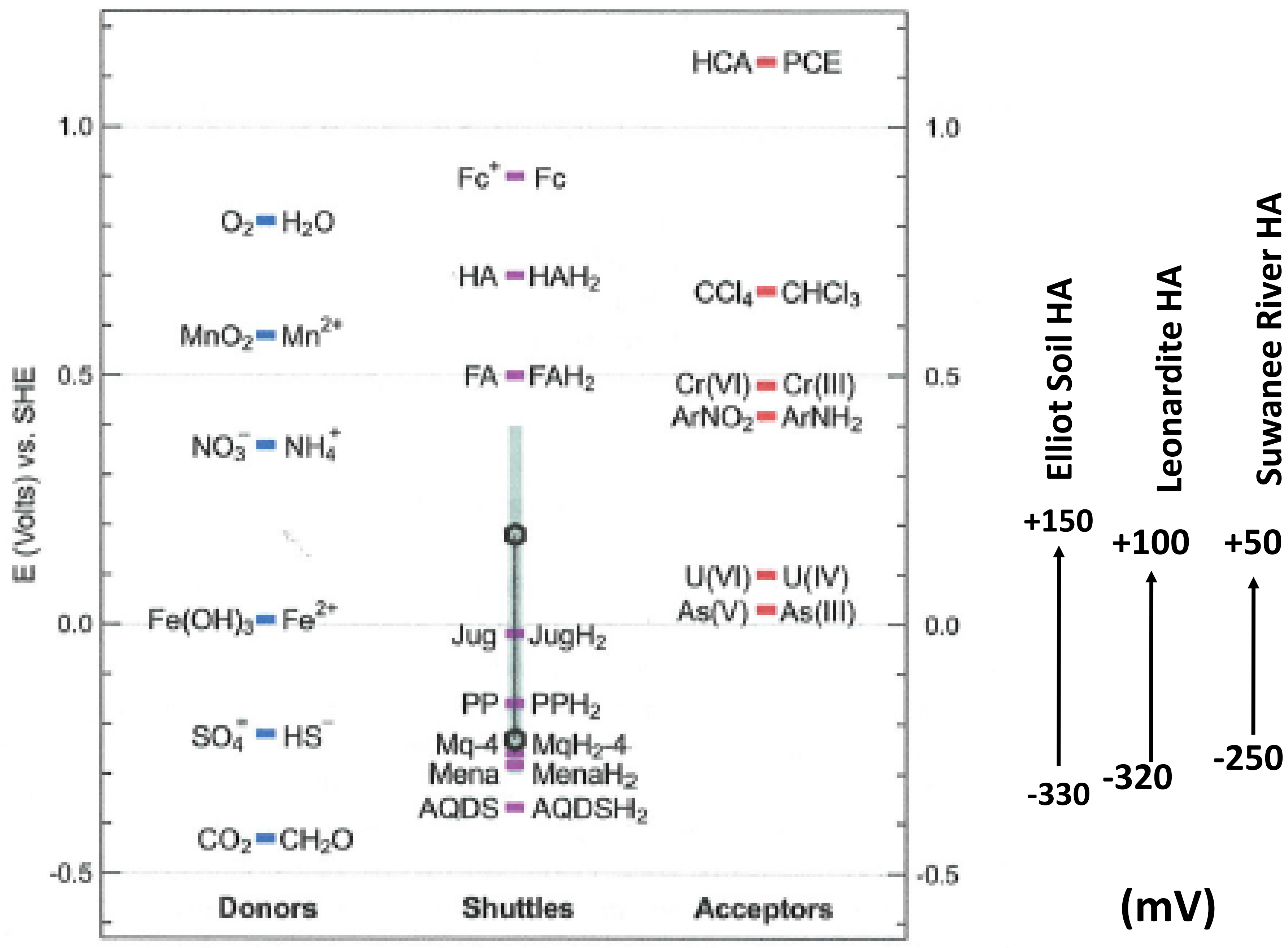
Free Radical Content and Electron Accepting and Donating Capacities of Humic Acids From Different Ore Sources

Humic acid	Free radical cont. (spins g^{-1})
NMHA	4.24×10^{17}
BH HA	1.92×10^{17}
UM HA	4.48×10^{17}
BE HA	1.85×10^{17}
NDL HA	4.21×10^{17}
ORYX HA	1.18×10^{17}
OMNIA HA	1.87×10^{17}

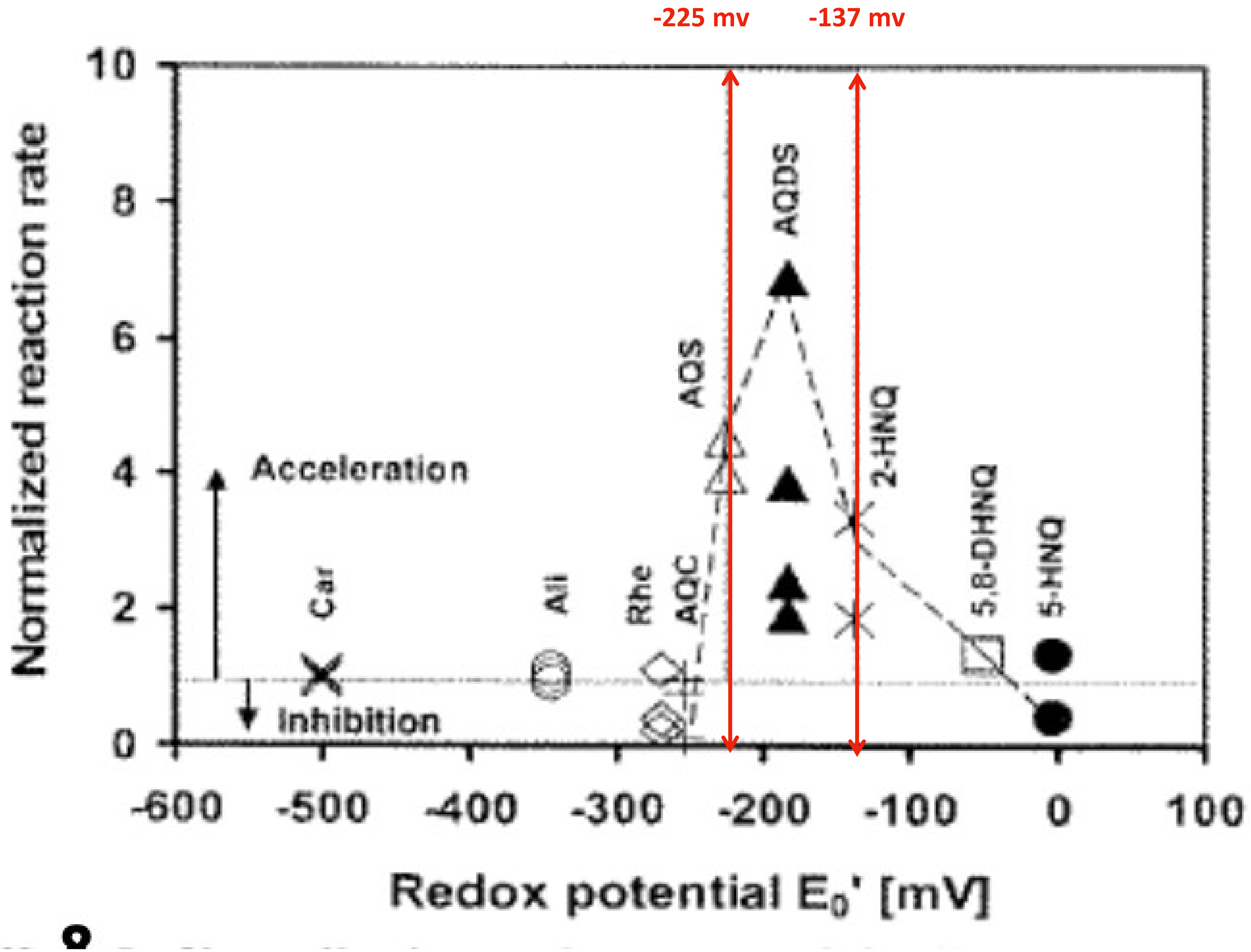
Humic acid	EAC (mmol g^{-1})	EDC (mmol g^{-1})
NM HA	2.54a	0.619de
BH HA	2.26bc	1.131b
UM HA	2.13c	0.594e
BE HA	2.48a	1.156b
NDL HA	2.38ab	0.753c
ORYX HA	1.70d	0.684d
OMNIA HA	1.61d	1.451a
VC	0.57e	0.620de

Relationship between EAC/EDC and % Increase in total fine root length (0 - 0.5 mm diam.) over control





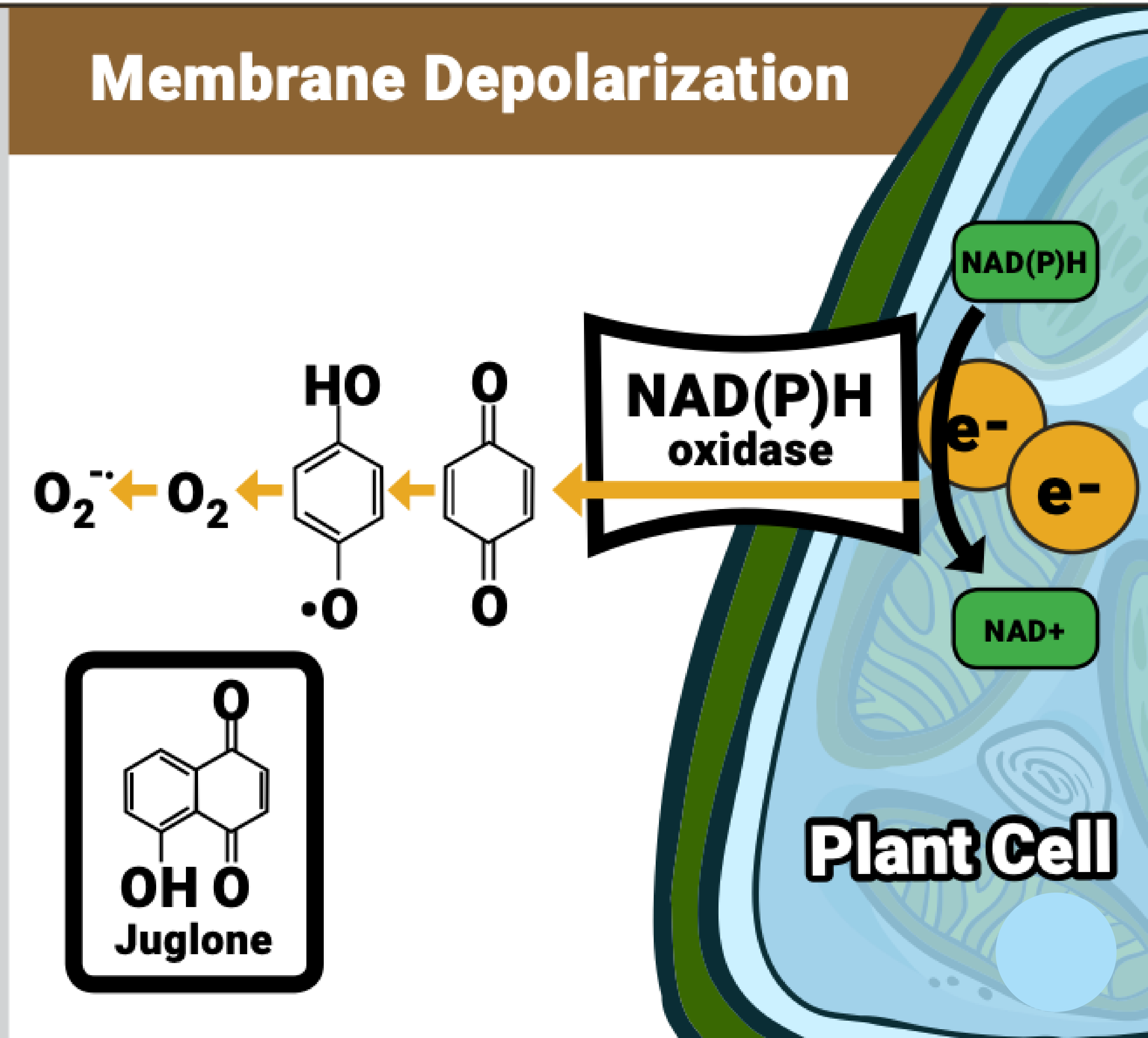
E (Volts) vs. SHE
1.0
0.5
0.0
-0.5
HCA PCE
Fc+ Fc
O2 H2O
HA HAH2
CCl4 CHCl3
MnO2 Mn2+
FA FAH2
Cr(VI) Cr(III)
ArNO2 ArNH2
NO3- NH4+
U(VI) U(IV)
As(V) As(III)
Fe(OH)3 Fe2+
Jug JugH2
PP PPH2
SO4= HS-
Mq-4 MqH2-4
Mena MenaH2
AQDS AQDSH2
CO2 CH2O
Donors
Shuttles
Acceptors
Elliot Soil HA
Leonardite HA
Suwanee River HA
+150
+100
+50
-330
-320
-250
(mV)



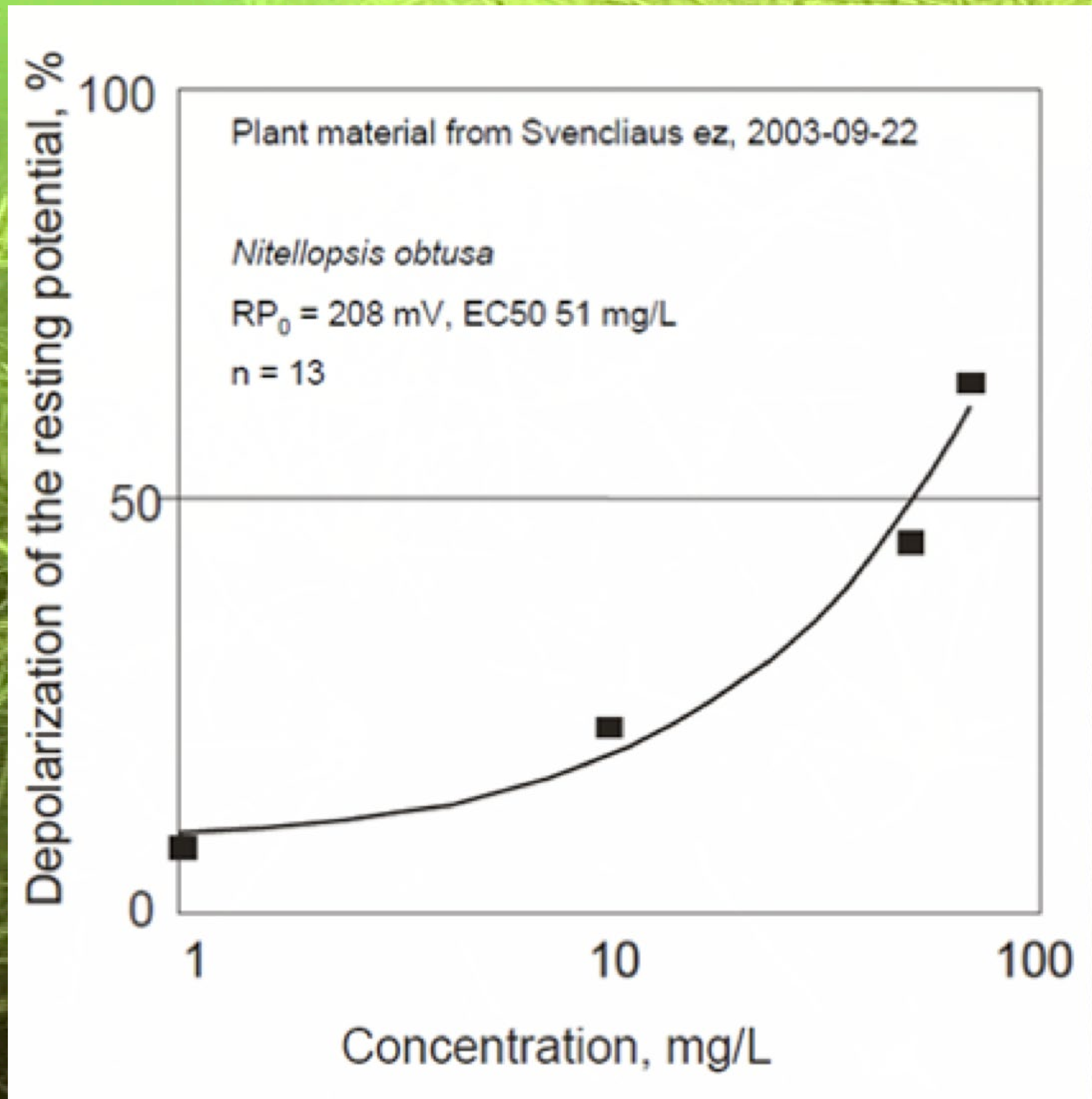
Effect of quinone redox potential on rate of ferrihydrite reduction by Geobacter metallireducens.

Superoxide Production By One-Electron Reduction Of Quinones *by PM NA(P)H Oxidase*

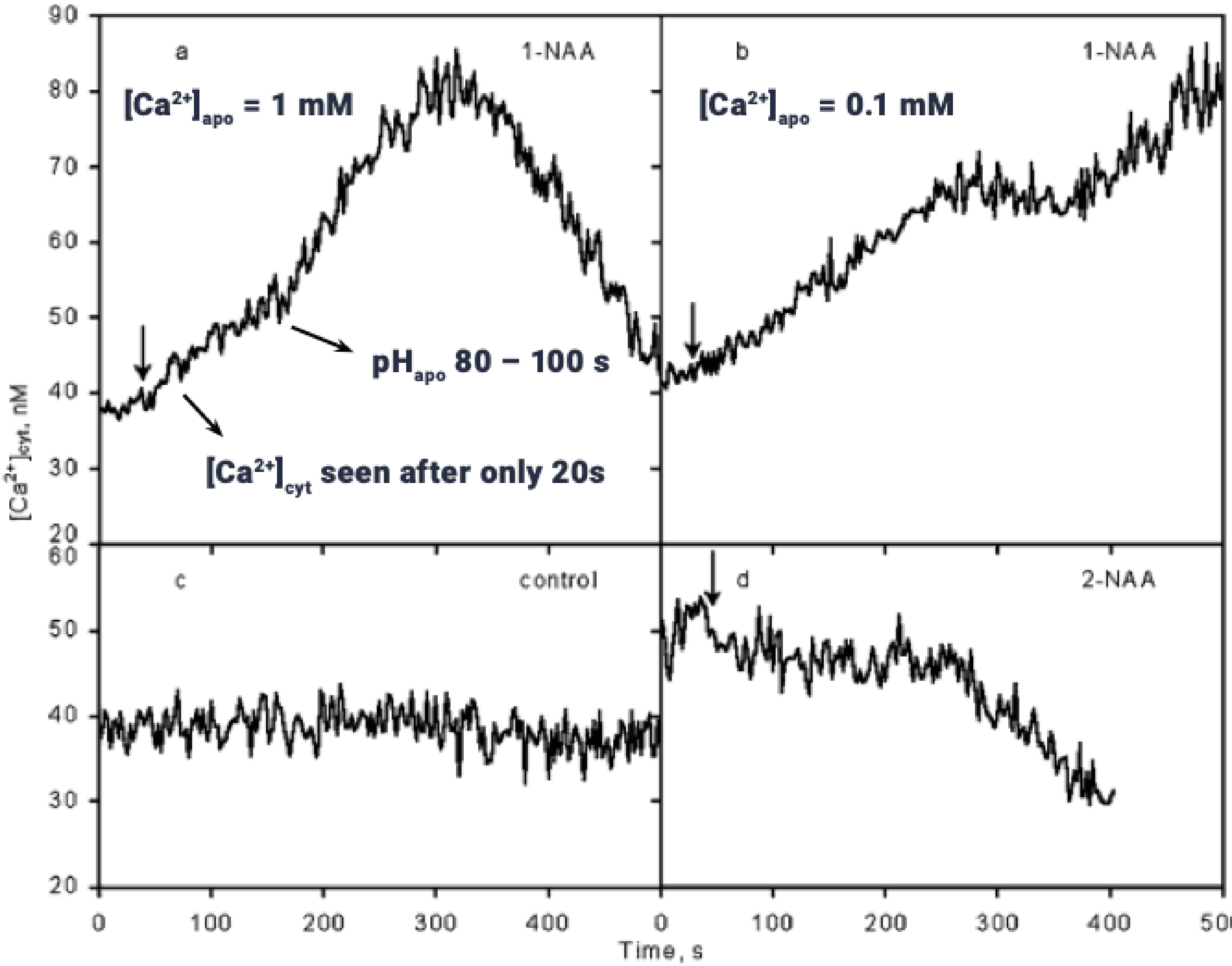
	Addition	NADH-Dependent	NADPH-Dependent
	PM alone	6.9 ± 1.8 (8)	8.2 ± 0.7 (16)
	Naphthoquinones (NQ)		
-93 mV	Juglone	169.3 (1)	160 ± 40 (4)
-203 mV	Menadion	70 ± 7 (3)	83 ± 7 (7)
	2.3-diCl-1.4-NQ	NT	92 (1)
	1,4-NQ	NT	49 (1)
-353 mV	2-OH-1,4-NQ	NT	8.86 ± 0.14 (2)
	Other Inducers		
	Duroquinone	NT	13 (1)
	Anthraquinonebisulfate	NT	7 (1)
	Fusarubin	NT	11 (1)
	Paraquat	NT	8 (1)



PM Depolarization by Humic Acid



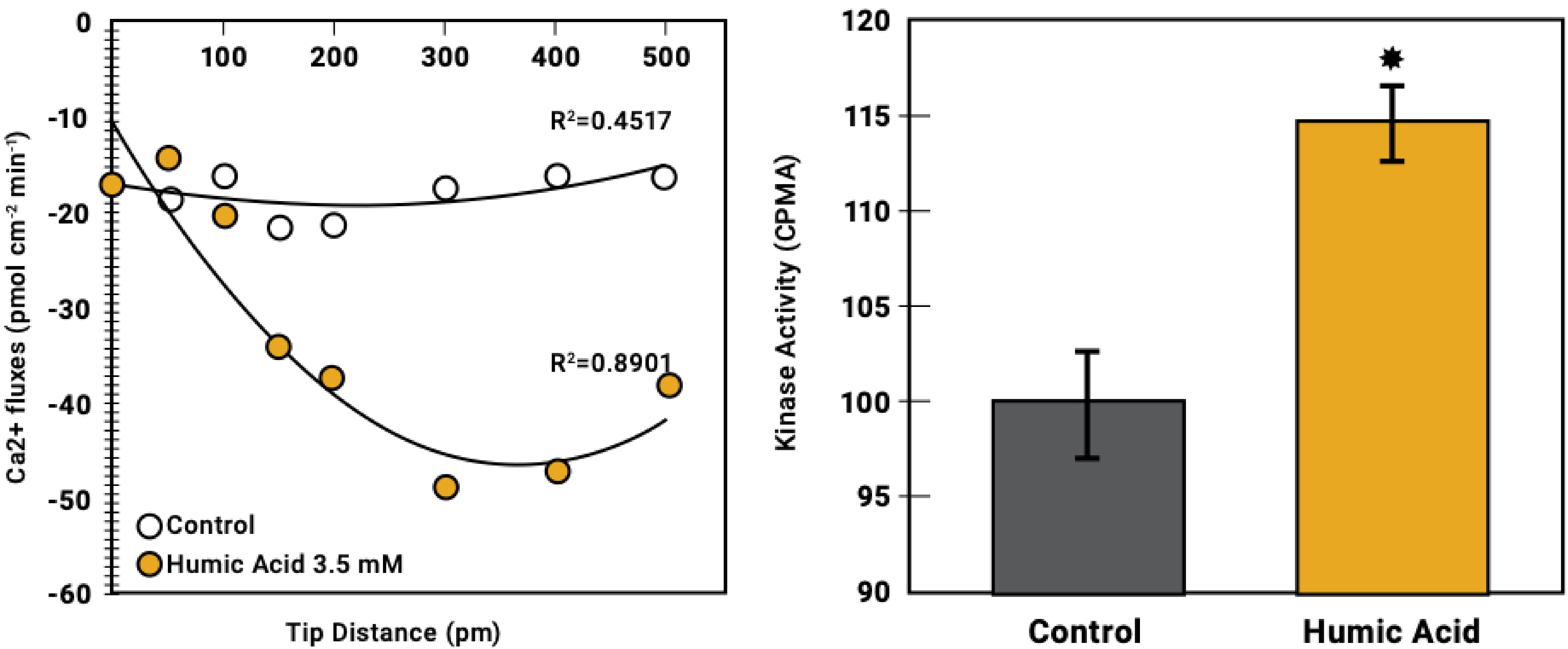
Effect of **auxin addition on $[Ca^{2+}]_{cyt}$** to wheat seedling protoplast



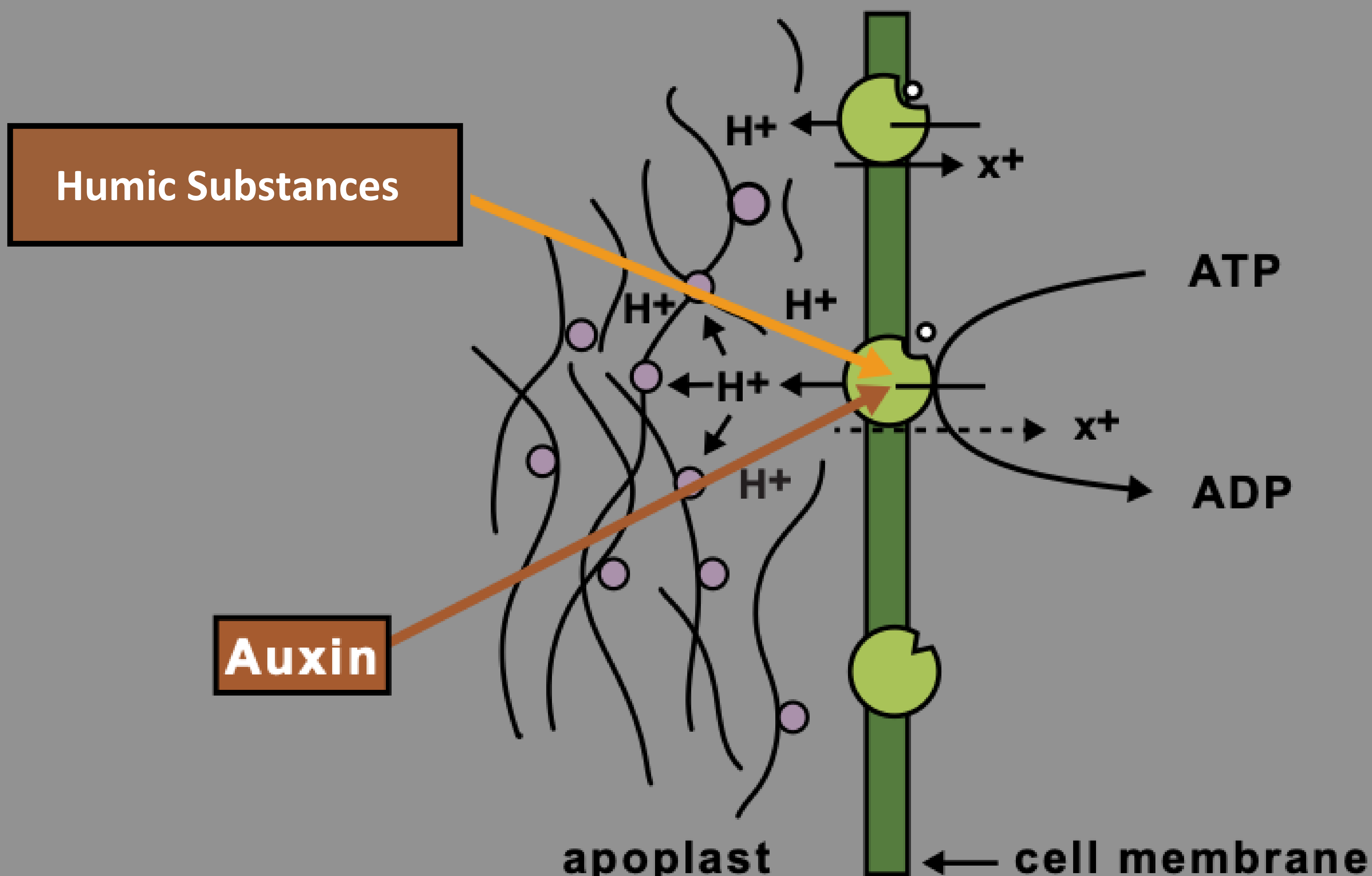
Shishova and Lindberg, 2004. Auxin induces an increase of Ca^{2+} concentration in the cytosol of wheat leaf protoplasts. J. Plant Physio. 161: 937 – 945.

Effects of Humic Acids

on root Ca^{2+} fluxes and expression of CDPKs

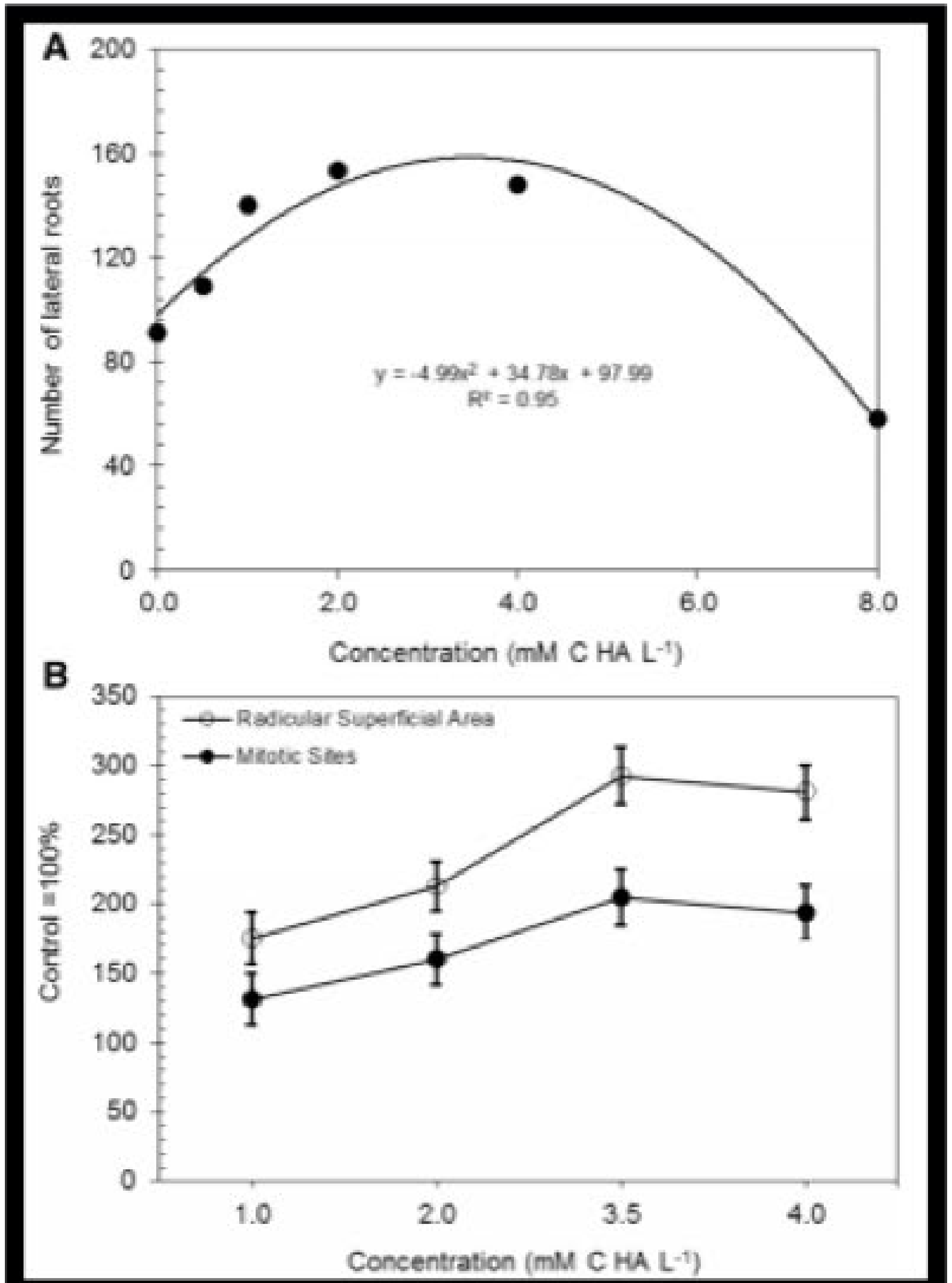


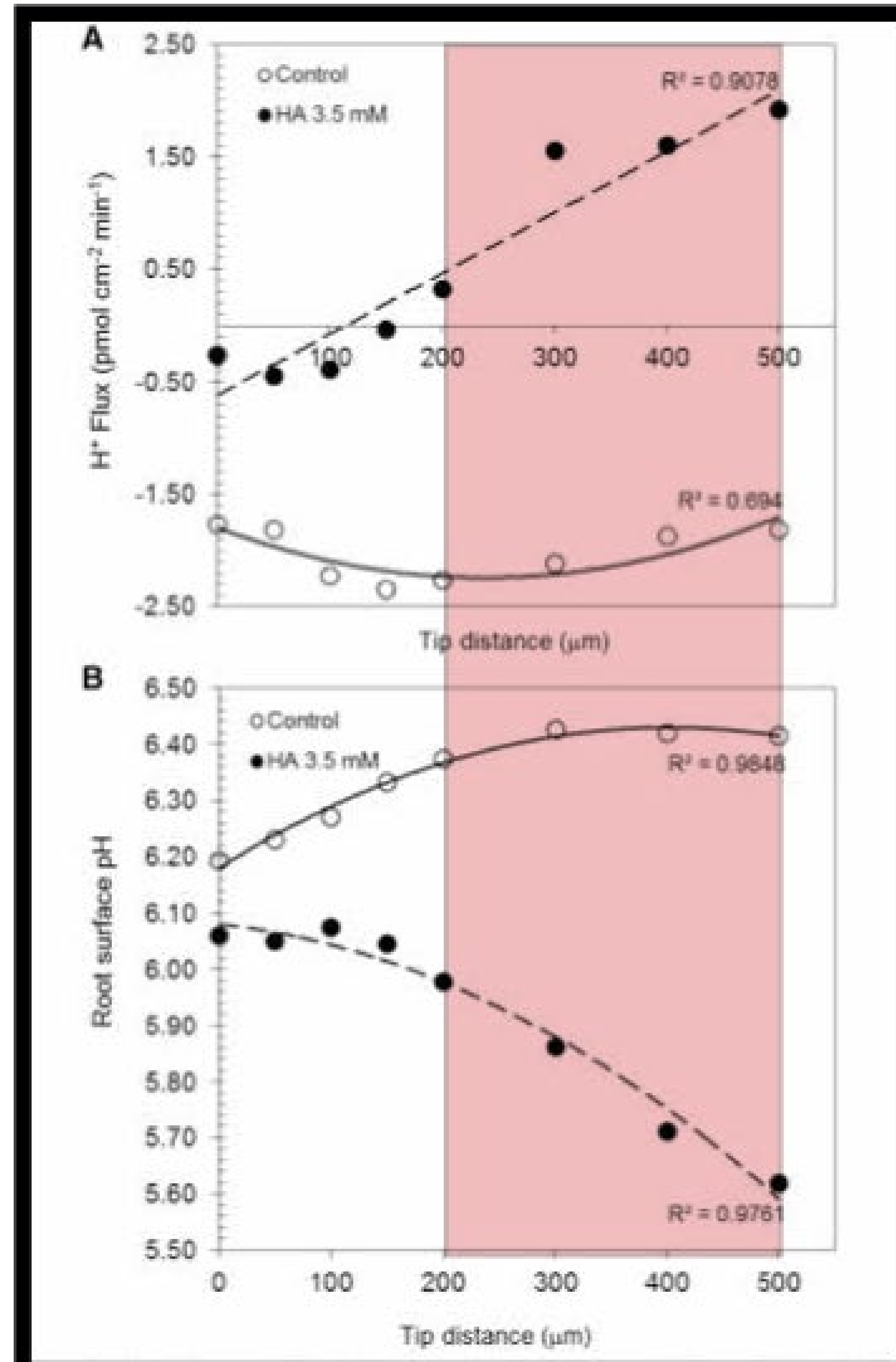
Humic Substances mimic the activation of H^+-ATPase activity of Auxin



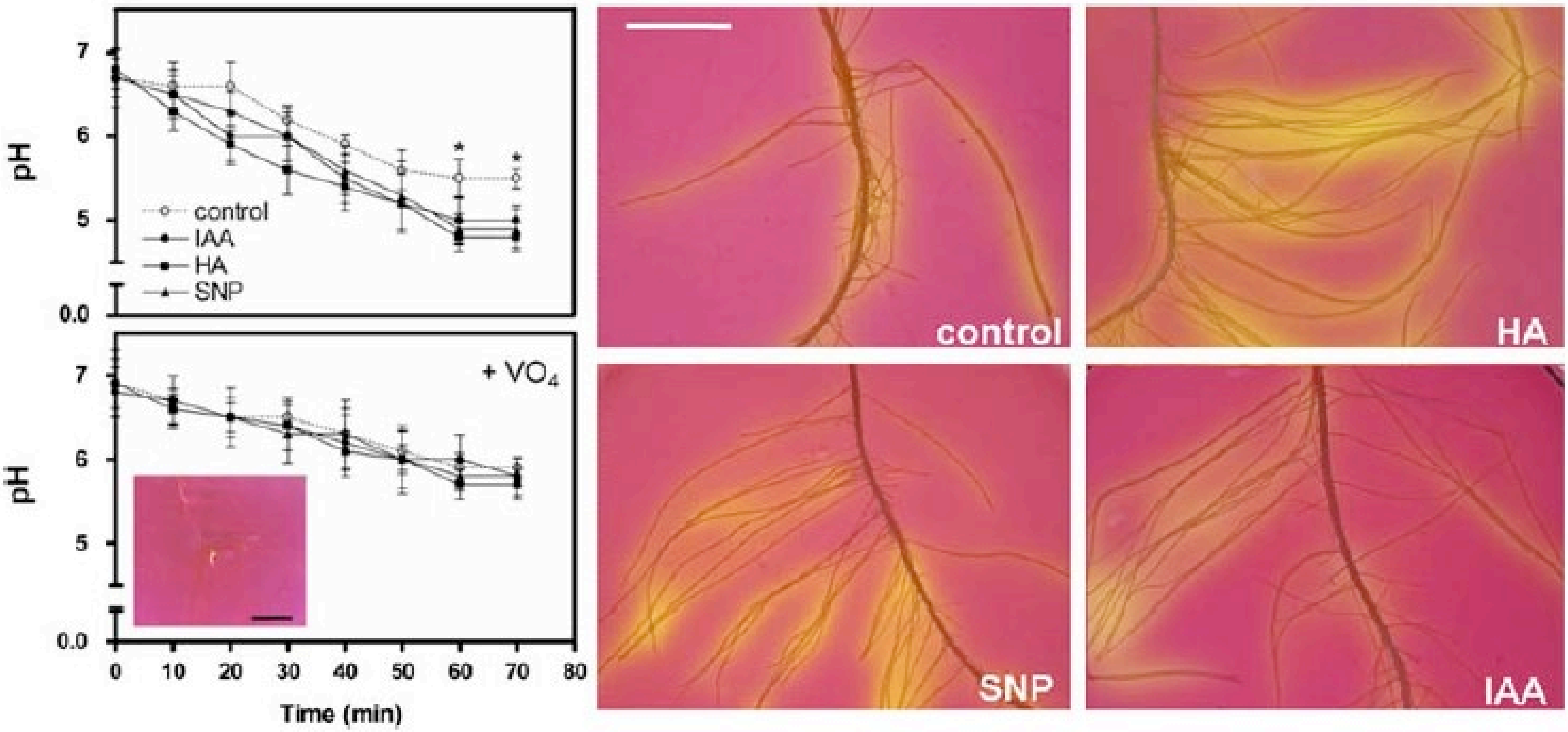
Effects of Humic Acids

on Numbers of Lateral Roots And H⁺ Fluxes From Roots





Bromocresol Purple Assay to Detect H+-ATPase Activity



Purple = pH > pH6 Yellow = pH < pH 6

Effect of humic acids (20 mg C L^{-1}) and IAA (auxin) on the activities of Maize PM H^+-ATPase and tonoplast H^+-ATPase and H^+-PPase

Table 2 Effect of HA from Ultisol (HAU), Inceptsol (HAI) sewage sludge (HAS) and vermicompost (HAV) or 10^{-5}, 10^{-10} and 10^{-15} M IAA on proton pumps

Treatments	Hydrolytic activities		
	Plasma membrane	Tonoplast	
	H^+-ATPase (µmol Pi mg^{-1} min^{-1})	H^+-ATPase (µmol Pi mg^{-1} min^{-1})	H^+-PPase (µmol PPi mg^{-1} min^{-1})
Control	1.57 ± 0.13 (C)	0.15 ± 0.02 (C)	0.13 ± 0.02 (C)
HAU	2.75 ± 0.14 (B)	0.41 ± 0.02 (A)	0.32 ± 0.02 (A)
HAI	4.65 ± 0.24 (A)	0.24 ± 0.03 (BC)	0.29 ± 0.04 (AB)
HAS	4.58 ± 0.12 (A)	0.32 ± 0.02 (AB)	0.19 ± 0.01 (C)
HAV	2.97 ± 0.13 (B)	0.40 ± 0.03 (A)	0.16 ± 0.02 (C)
IAA 10^{-5} M	1.19 ± 0.17 (C)	0.23 ± 0.03 (BC)	0.17 ± 0.01 (C)
IAA 10^{-10} M	2.52 ± 0.12 (B)	0.33 ± 0.01 (AB)	0.18 ± 0.01 (C)
IAA 10^{-15} M	2.41 ± 0.10 (B)	0.17 ± 0.01 (C)	0.22 ± 0.01 (BC)

Conclusions

* The initial metabolic responses of plants to HS are identical to those in plants experiencing stress.

* It is the intensity of the stress that governs whether the response will result in eustress (beneficial stress) or reduced plant health and possibly death.

* Quinones in HS can act as electron shuttles and may be one of the agents responsible for elicitation of a of mild stress response in plants in concert with antioxidant phenolic hydroxyls.

* Quinones can act as extracellular electron acceptors for enzymes (e.g., NADPH oxidases) involved in transmembrane electron transport.

* Therefore, quinones in HS can most likely also initiate transmembrane electron transport in plant cells resulting in a eustress response.

* Strong correlation between EAC/EDC (i.e., pro-oxidant/antioxidant) in a number of diverse HA and stimulation of plant fine root growth supports the HA quinone electron shuttle theory.