



AGEING PHYSIOLOGY IN TERMS OF ROOTING RESPONSE : RESEARCH REVIEW

Abdullah Ibrahim Shaheed

Department of Biology, College of Science, University of Babylon, Iraq.

Abstract

At the mid of the 1990s a novel program has been started, including an experimental system represented by mung bean (*Phaseolus aureus* Roxb.) cuttings in terms of rooting response, aimed to- 1) verification of all hypotheses (ten) that explain ageing causes, which already suggested in different experimental system and different spp. 2) Assessment the possibility of occurring, ageing processes that deal with above hypotheses in the same experimental system, keeping in mind the priority of a process over the others. 3) Attempts for controlling (delaying) the processes that occur during ageing by physico-chemical means in terms of maintaining the cutting's sensitivity for subsequent indicative auxin treatment. Some of these attempts are failed, some succeeded partially and others succeeded completely. 4) A new hypothesis (reversed Na^+/K^+ pump action hypothesis) was suggested in this study.

Key words :Ageing, anti-oxidants, blockage of xylem vessels, boron, CAT, chemical potentiation, IAA, permeability perturbation, rooting response, Na^+/K^+ pump, Subrin, SOD and Tyloses.

a) Ageing Physiology

Ageing is a phenomenon, having different aspects on the morphological, physiological, social, psychological (behavioural), cellular and molecular levels, any definition involved all of the above aspects is impossible (Davis, 1983). The latter, however, described ageing as the failure of maintaining the stability of endogenous environment (homoeostasis) under physiological stress. Such failure is associated with a decline in viability and increased the vulnerability of an individual (Comfort, 1979).

Generally, multi-definition has been given to describe ageing whether on the whole plant, or cellular level (Woolhouse, 1967). Some of these concepts are:

First, biochemical and genetical changes that lead to the gradual senescence and death of cell or organism (Coombs, 1986).

Second, decline or loss of plant tissue response for auxin (Salisbury and Ross, 1992).

Third, ageing is a phenomenon characterised by being universal, progressive, intrinsic and degenerative. However, only the final criterion can be used to separate

ageing from age-related developmental and maturation processes (Strehler, 1962).

Fourth, ageing was described by degradation of DNA, RNA protein and chlorophyll as a result of increasing the activity of enzymes: DNAase, RNAase, protease and chlorophyllase respectively (Ables, 1966) as well as phospholipid degradation due to the activity of Lipoxigenase enzyme (Shaheed *et al.*, 2009a).

Finally, in terms of adventitious root formation (ARF) ageing was described as all processes that lead to diminishing rooting response in mung bean (*Phaseolus aureus* Roxb) cuttings by delaying the inductive auxin treatment (keeping cuttings in deionized water for a certain time (ageing period) (Jarvis and Shaheed, 1986).

b) Hypotheses that explain ageing causes

Ten hypotheses have been postulated to explain ageing causes in the different experimental system, these are:

- (1) The decline of naturally occurring auxin (IAA) (Hartmann *et al.*, 1988).
- (2) Blockage of xylem vessels due to the formation of waxy materials such on suberin or cutin (Cline

and Nelly, 1983).

- (3) Permeability perturbation of cytoplasmic membranes (Legal *et al.*, 1982).
- (4) Oxidative hypothesis (Gorecki *et al.*, 1991), the formation of high levels of reactive oxygen species (ROS) and subsequently causing the member damage.
- (5) Shortage (deficiency) of nutritional status (Molisch, 1938; Sklensky and Davis, 1993).
- (6) The decline of rooting co-factors (Wally *et al.*, 1980).
- (7) Callose formation and bulging of phloem sieve plate (Ulrich, 1962).
- (8) Increasing the activity of IAA oxidase enzyme (Chibbar *et al.*, 1979).
- (9) The decline of phenolic compounds and its role as an auxin-protectors (Zenk and Muller, 1963).
- (10) Increasing of abscisic acid (ABA) level (Atkinson *et al.*, 1989).

c) Ageing in terms of adventitious root formation (ARF)

Jarvis and Shaheed (1986) have been studied the influence of ageing on rooting response, uptake of basally supplied auxin and acropetal transport and distribution of C^{14} IAA, basipetal transport of C^{14} -IAA after its application to primary leaves and transpiration loss in mung bean cuttings, the result revealed that : Freshly prepared cuttings treated with IAA, 5×10^{-4} M produced 75 roots, when treatment with IAA was delayed by ageing the cuttings in deionized water for an intervening period, rooting progressively declined. After ageing for 1d an average cutting, produced approximately 46 roots whereas after 4d mean root number per cutting was only 12. The longer the delay in supplying exogenous auxin the smaller is the rooting response.

This work has made use C^{14} -IAA to establish quantitatively the uptake and distribution of basally-supplied auxin in fresh and aged cuttings. In addition, the transport of C^{14} -IAA from leaves to hypocotyl has been assessed. It has been demonstrated that changes in uptake, transport and metabolism are associated with the progressive loss of responsiveness to supplied IAA, which is observed when cuttings are aged in water.

Cuttings aged in water show a progressive decline in transpiration and an associated decrease in uptake of basally supplied IAA. However, this uptake is essentially passive via the transpiration stream. This is a novel observation which contradicts the suggestion of Batten

and Goodwins (1981) that auxin enters the hypocotyl through the epidermis. In that investigation, however, auxin uptake was studied only into isolated hypocotyl (in absence of leaves). However, the diminished uptake of auxin-associated with increasing age is unlikely to be the only factor influencing the rooting response in aged cuttings. Jarvis and Booth (1981) suggested that the leaves act as centres to control not only the uptake of supplied auxin but also its subsequent loading into the appropriate basipetal transport system. This implies that rooting due to supplied auxin involves acropetal transport of auxin to the leaves followed by its subsequent basipetal transport. If the amount of auxin in the hypocotyl is the limiting factor for root formation, the decline on both acropetal and basipetal transport associated with increasing ageing (tables 1 and 2) would each contribute to the decline in root production. However, calculations based on data of uptake of basally supplied C^{14} -IAA and distribution through the cutting, show that after ageing the total amounts of supplied auxin which accumulated in the hypocotyl were greater than in hypocotyls of fresh cuttings, yet rooting was less in each case. This establishes an important point – namely that there is no correlation between the number of roots regenerated and the total amount of auxin accumulated in the hypocotyl. Either some factor(s) other than auxin control(s) rooting or alternatively only that auxin, which is basipetal transported into the hypocotyl is important for root formation. On the basis of the work described here one cannot distinguish between these observations.

d) Verification of ageing hypotheses

To verify the hypotheses that already conducted in different experimental systems which explain ageing causes, Shaheed at the mid of the 1990s started a novel program including one experimental system represented by mung bean cuttings, aimed to :

1. Verify of the above hypotheses.
2. Test the possibility of occurring different ageing processes that deal with above hypotheses in the same tested experimental system.
3. Controlling the processes that occur during ageing by physicochemical means in terms of maintaining the cutting's sensitivity for subsequent inductive auxin treatment.

Thus, an experimental system that employed in this program was represented by mung bean cuttings (*Phaseolus aureus* Roxb.) were taken from 10-day-old light-grown seedlings (Hess, 1961). Seedlings were raised in growth cabinet provided with a continuous irradiation supplied by worm white fluorescent tubes (1500-1800

Table 1 : The influence of ageing on the percentage distribution of ^{14}C -IAA in stem cuttings. Cuttings, with or without prior ageing, were supplied basally with IAA ($5 \times 10^{-4}\text{M}$, $59.2 \text{ MBq mmol}^{-1}$) for 4 h, after which time the radioactivity in various parts of the cuttings was determined.

	Time (d) for which cuttings aged				
	0	1	2	3	4
Leaves and terminal bud	24.7	17.4	8.7	6.7	5.5
Epicotyl	55.8	53.1	45.0	45.8	40.0
Hypocotyl	19.4	29.5	46.3	47.6	54.5

Table 2 : The influence of ageing on the recovery and transport of ^{14}C -IAA applied to the leaves of cuttings. Cuttings were aged in water for the various period before application of ^{14}C -IAA to the leaves (7.4 kBq to each leaf). Distribution of radioactivity within the cuttings was determined 24 h later.

Bq recovered from	Time (d) for which cuttings aged				
	0	1	2	3	4
Leaves	5,553±1,187	5,021± 949	4,867±857	3,413±409	3,087±413
Terminal bud	20.2 ± 7.1	12.1±4.9	3.8±1.2	2.3 ±1.0	2.0 ± 0.8
Epicotyl	44.7±7.0	40.3±11.4	8.5±0.6	5.5±2.0	5.2±1.9
Hypocotyl	29.7±7.3	26.9 ± 9.5	4.9±3.3	1.6±0.4	1.0 ± 0.3
Whole cutting	5,648	5,100	4,884	3,422	3,095
Percent of total (recovered) radio activity located in hypocotyl, epicotyl and bud	1.67	1.55	0.35	0.27	0.26

Table 3 : Influence of medicinal herb extracts on ageing of mung bean cuttings, not induced by auxin.

Ageing treatment for 3days in:	Subsequent treatment For 24h in:	Mean Root No./cutting for 6days in borate
d/H ₂ O	None	8.4
d/H ₂ O	IBA10 ⁻⁴ M	24.9
Anise, 1%	None	25.1
Negella, 1%	None	22.1
Milfoil, 1%	None	18.7
Fenugreek, 1%	None	24.3
Common nettle, 0.1%	None	10.3

lux, 400-700nm), temp. $25 \pm 1^\circ\text{C}$ and relative humidity 60-70%. Stem cuttings consisted of small terminal bud, a pair of fully expanded primary leaves, a whole epicotyl and hypocotyl (3-cm length) under cotyledonary nodes. Cuttings were held during all treatments under the same conditions that used to raise seedlings.

e) Decline of naturally occurring auxin (IAA) hypothesis

To verify this hypothesis, Shaheed and Al-Alawi (2001) was found a decline (%65) in rooting response of

aged cuttings (by holding in distilled water) when treatment of cuttings with inductive auxin treatment (IBA, 10^{-4} M) is delayed. This decline was coincided with reducing IAA level in aged (primary leaves, epicotyl and hypocotyl) compared to fresh cuttings. Meanwhile, cuttings kept in Anise (*Pimpinella anisum* L.) extract a higher level of IAA compared to those held in d/H₂O.

As an attempt to delay the processes that occur during ageing, cuttings were treated for 3-days with 8 medicinal herb extract instead of d/H₂O. Data shows that 5-out of these herbs were able to delay or stop completely the process that occurs during ageing. These herbs are Anise (*Pimpinella anisum* L.), Fenugreek (*Trigonella foenum graecum* L.), Milfoil (*Achillea sentonlina* L.), Negella (*Nigella sativa* L.) and common nettle (*Urtica dioica* L.) at conc. Of 1% except 0.1% for common nettle. The mean root no./cutting for these herbs are (55, 47.8, 49.6, 51.5 and 48.4) respectively with no significant difference (P 0.05) among them compared to fresh cuttings that treated with auxin (54.8 roots). In other words, aged cuttings that kept in extracts of the above herbs response to auxin treatment after 3-days as it was the case in fresh cuttings.

It is noteworthy that medicinal herbs at concentrations active in delaying ageing have themselves had no influence

on root development (table 3) unless cuttings are supplied with inductive auxin treatment.

An additional result of this study confirmed the above idea and indicated that herb extracts had no effects on ARF in fresh cuttings when supplied for 24h as an alternative to auxin except Anise and fenugreek. The later developed 23.2 and 26.3 roots compared to control treatment (distilled H₂O = 6.3). Such rooting responses represent < %24 compared to fresh cuttings supplied with auxin (62 roots). These results confirm, the possibility of employing water extract of medicinal herbs as an alternative of plant growth regulators in ARF, especially when compared to low concentrations of auxin (less than 10⁻⁴M) in which the effect may be equal.

Cuttings aged in d/H₂O and treated with auxin for 24h then transferred to boric acid for further 6d. In addition, cuttings aged in d/H₂O or medicinal herb extracts and transferred directly to boric acid for 6d. LSD = (0.05 = 5.65, 0.01 = 7.49).

Data presented in this study raises the main point that is, a decline of naturally occurring auxin (IAA) in aged cuttings particularly in leaves which consider as a site of IAA biosynthesis. Ageing is fundamentally concerned with degenerative changes in metabolism (Davies, 1983). The latter author raises the possibility of alteration of hormonal balance as the only molecular events leading to these changes.

Extractable auxin content in aged cuttings is lower than in fresh cuttings, particularly in primary leaves. This decline in IAA content may be attributed, at least, to one of the following possibilities:-

First : The decline of auxin biosynthesis in primary leaves of aged cuttings, though it was the main site of its synthesis was agreed with (Hartmann *et al.*, 1988) in senescent leaves, in fully expanded leaves (Wilkins, 1975) and primary leaves of older (aged) seedlings (Jarvis and Booth, 1981).

Second : Increase of IAA-oxidase activity in aged tissues, which leads to the destruction of auxin (IAA) (Morgan, 1964).

Third : Conversion of free (extractable form) to bound auxin (conjugated form) in aged cuttings (Norcini, *et al.*, 1985).

Fourth : A decline in the rate of basipetal transport of auxin in aged cuttings (Shaheed & AL-alwani, 2001).

f) Blockage of xylem vessels hypothesis

Ageing, causes and control in terms of ARF in mung bean cuttings have been studied physiologically,

anatomically and histochemically by (Shaheed and Al-Alwani, 2002). This novel investigation involves verification of a hypothesis that deals with blockage of xylem vessels in aged cuttings. Furthermore, the ageing (blockage of xylem vessels) was controlled by using an aqueous extract of medicinal herbs (*e.g.* Anise leaves 1%).

The results revealed a decline in transpiration loss of aged cuttings compared to fresh cuttings. Such decline was correlated with the blockage of xylem vessels and coincides with the average (speed) of blockage and timing of its appearance in terms of suberin formation.

However, the blockage was occurred chemically as a result of suberin formation in the basal centimetre of the hypocotyl and move acropetally (results of histochemical part of the study) but not by tyloses formation (results of anatomical part of the study). Consequently, such blockage interrupts transpiration flow and causes retardation of acropetal movement of basally supplied auxin toward the leaves, hence, its re-distribution to the hypocotyl (root initiation zone).

The control of ageing phenomenon was achieved by overcoming (offsetting) the blockage of xylem vessels in aged cutting by its treatment with aqueous extract of Anise (1%) during ageing periods. It might be attributed to the hydrolysis of suberin that formed in the basal segment of aged cuttings and the inhibition of suberin biosynthesis in the middle and top segments of the hypocotyl.

It is well known, that excision of the basal cut surface of the cuttings induce the formation of tyloses, but the anatomical results revealed opposite situation. Seemingly, the disappearance of tyloses does not mean absolutely it is not formed because of the chemical blockage by suberin. In other words, the suberin occupies the xylem vessels internally (within less than 4h), and prevent the development of tyloses by closing the pits in the xylem vessel walls.

g) Permeability perturbation hypothesis

Shaheed and Jabor (2009a) has been studied the effect of ageing on permeability perturbation (P.P) in terms of rooting response, depending on the decrease of protein and phospholipid levels as an indicator of P.P of cytoplasmic membranes during ageing phenomenon. In addition, to the percentage of electrolytes leakage in terms of electrical conductivity and average of ions efflux such as Ca⁺², Mg⁺² in different parts of cutting. Moreover, controlling the processes that occur during ageing by using aqueous parsley (*Petroselinum crispum* Mill) seed extract in terms of ARF via the above parameters.

Effect of ageing on chemical structure of membrane

It is well known that the chemical constituents of cytoplasmic membranes are mainly proteins and phospholipids. The configuration (architecture) of membranes depend on these materials and its organisation in special model as described by Singer and Nicolson (1972) to give the fundamental property of membrane that called "permeability". Changes in the average of fluxes (influx and efflux) considered as indicators for permeability perturbation. The latter caused by two ways; firstly shortage in protein and/or phospholipid levels. Secondly alteration or modification of the membrane configuration. Possibly, both ways could happen due to the shortage of proteins and phospholipids.

Ageing caused a decline in protein content (21%) of cuttings aged in distilled water compared to fresh cuttings. There is an agreement about the relationship between ageing and the decline in protein content in aged barley and bean seeds (Mudgett *et al.*, 1997; Michalczyk *et al.*, 1998). This decline may be attributed to increasing of protease activity during ageing (Shaheed *et al.*, 2009a). Meanwhile, the decline in total phospholipids content in aged cuttings (>50%) are in agreement with previous studies such as flower tissues of *Ipomea tricolor* by (Beutelmann and Kend, 1977), roots of cassava (Lalaguna and Agud, 1988) and seeds of *Vicia faba* (Michalczyk *et al.*, 1998). However, the decline in phospholipid level may be attributed to increasing the activity of lipoxygenase (Shaheed *et al.*, 2009a) or phospholipase (Hopkins and Huner, 2009) in addition to the decline in its biosynthesis or both. Alternatively, the reason may be associated with free radicals that increase during ageing (Gorecki *et al.*, 1991) and causes lipid peroxidation thereafter, creates permeability perturbation.

The influence of parsley extract in controlling ageing (permeability perturbation)

Usage parsley extract (1%) to control ageing instead of distilled water for 3 days shows a decline in the percentage of K^+ and Mg^{+2} ions leakage (increasing of membrane repair) in primary leaves, epicotyl and hypocotyls (36.07, 58.63 and 78.02), respectively. Keeping cuttings in parsley extract (1%) for 3 days enhances retention of cuttings sensitivity to subsequent auxin treatment (IBA, $10^{-4}M$). Surprisingly, in terms of ARF, these cuttings respond (57.58 roots/cuttings) as it was the case in fresh cuttings (57.9 roots/cuttings) by reducing the statistical differences between them (completely offsetting ageing processes).

On the other hand, the above extract does not significantly induce more adventitious roots on aged

cuttings unless IBA was supplied (27.91 roots in extract compared to 22.75 roots in distilled water). Obviously, the role of extract resides in controlling the processes that occur during ageing phenomenon more than ARF.

Seemingly, parsley extract retards the perturbation that occurred in membrane permeability through repairing of the membrane or maintaining it is the constitution of protein and phospholipids. This effect may be attributed to the presence of phenolic compounds in parsley extract (preliminary tests). Harborne (1993) has mentioned that phenolics have the capability of forming complexes with proteins by hydrogen bonds, as a mechanism for repairing membranes. Shaheed (1997) confirmed this idea by stopping ageing completely in terms of ARF in mung bean cuttings by using high concentration ($10^{-3} M$) of cinnamic acid. However, phenolics (*e.g.* caffeic acid) has a role in raising IAA level that required for adventitious roots initiation by acting as auxin-protectors via its inhibitory effect for IAA- oxidase (Chibbar *et al.*, 1979).

In addition, the influence of parsley extract in promotion of rooting response in aged cuttings may be attributed, to the following reasons: (a) Implication of Ca^{+2} in the extract (Chakravarty, 1976) as co-factor in ARF (Hartmann *et al.*, 1990) (b) The role of Ca^{+2} in maintaining the physical integrity of membranes or its repairs (Epstein, 1972) and (c) The presence of substances acting as anti-oxidant agents through oxidative hypothesis, which is one of the hypotheses that explain ageing causes (Gorecki *et al.*, 1991). These substances (*e.g.* sucrose or phenolic compounds such as o-coumaric acid, caffeic acid and p-hydroxyquinone) has been found to delay completely the processes that occur during ageing of mung bean cuttings (its involvement in anti-oxidant defence mechanism) through maintaining IAA levels (Shaheed *et al.*, 2010a).

On the other hand, the influence of ageing on the percentage of ion influx (uptake) such as K^+ , Na^+ , Mg^{++} and Ca^{++} in terms of total content per a whole cutting, irrespective of its distribution through cutting parts has been studied by Shaheed and Jabor (2012). The latter shows that % of the above ions in the whole fresh cutting (as initial amounts) is as follows $K^+ < Na^+ < Mg^{++} < Ca^{++}$. Precisely the % of K^+ is two folds than Na^+ , 5folds than Mg^{++} and more than 6folds than Ca^{++} . However, in aged cuttings % of K^+ was declined to less than %50 while, % of Na^+ increased (44.9%) compared to fresh cuttings. In other words, decreasing % of K^+ and increasing % the of Na^+ making the equation become inversed and not agreed with the mechanism of the Na^+/K^+ pump action. So we suggested a new hypothesis acting reversibly in favourite

of Na⁺ influx, called (*Reversal Na⁺/K⁺ pump action hypothesis*). The new balance (distortion) explain ageing causes.

h) Oxidative Hypothesis

Most studies (e.g. Halliwell and Gutteridge, 1989; Gorecki *et al.*, 1991; Scandlios, 1993; Blokhimaes, 2000; Blokhima *et al.*, 2003) confirms what was previously reported by Harman (1959) about the oxidative hypothesis that explains ageing causes. This Hypothesis involved increasing the ROS formation during ageing of plant tissues that causes loss or decline of their responses (e.g. adventitious root formation) because of declining auxin content (Kerstetter and Keitt, 1966). Alternatively, the hypothesis involved a decrease in anti – oxidant defence agents, that needs activation by physical – chemical means (see an item I).

To verify the oxidative hypothesis in term ARF, three-approaches has been employed:

I) metabolic change, of lipid and proteins during ageing of mung bean cuttings.

II) Non-Enzymatic anti - oxidant defence mechanisms.

III) Enzymatic anti - oxidant defense mechanisms.

I) Metabolic changes of lipid & protein during ageing :

Recently, Shaheed and Jabor (2009a) found a decline in phospholipid (50%) and protein (21%) in aged mung bean cutting that causes Permeability perturbation. Consequently, shaheed and his colleagues (2009 a) shows that cuttings aged for 3-days in d/H₂O leads to diminishing rooting response (62.3%), which coincided with high level of oxidative processes occurring during ageing (The ageing was considered as physiological stress (Al-Hasnawi, 2012) because of: a) The availability of oxidative factors that increase malondialdehyde (MDA= final product of lipid peroxidation) due to increasing of lipoxygenase (LOX) activity (277%). b) Declining of protein due to the free radicals (an oxidative factors) or as a result of increasing Protease activity (212%). c) Declining of the non- enzymatic anti- oxidant defense factors such as total ascorbate and glutathione that reduced by 66.8% and 41.43%, respectively compared to fresh cutting (control). The above results were agreed with previous studies that deals with increasing the activity of hydrolyzing enzymes such a Lipoxygenase and protease (Kar and Mishra, 1976).

For controlling the Processes that occurs during ageing, (Shaheed *et al.*, 2009 a) reported that keeping cuttings for 3-days in Anise (*Pimpinella anisum*) aqueous

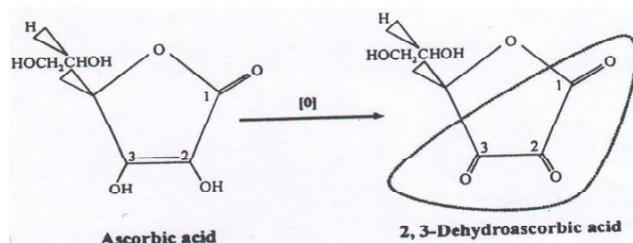
extract (1%) or Cinnamic acid (10⁻³M) maintaining the sensitivity of cuttings to inductive auxin treatment, that develops 48 roots / cutting without statistical difference compared to fresh cutting (50.9 roots). The latter case should he explained via retarding or delaying at least one of the processes that occur during ageing such as lipid/ protein peroxidation. Physico – chemical methods that employed in controlling ageing revealed biochemically decline in MAD via partial inhibition of at least LOX activity, and maintaining protein content (lowering protein hydrolysis) in aged cutting. In addition, maintaining the anti- oxidant level such as ascorbate & GSH which acts as scavengers of free radicals. Alternatively, Anise extract contains active substances as revealed by a preliminary test such as phenolics & terpenoids, acting as scavengers (Harborne, 1993), that able to protect cell components.

II) Non-enzymatic anti-oxidants

Depending on number and position of hydroxyl groups of some phenolic compounds, ascorbate and sugars, and their effects on IAA levels through oxidative hypothesis that accompanied ageing phenomenon of mung bean cuttings were studied by (shaheed *et al.*, 2010a). They found significant increase (P_{0.01}) in rooting response of cuttings aged in *o*-coumaric, caffeic acid and *p*-hydroquinone all at 10⁻³ M, ascorbate at 200-500 ppm and sucrose at 3%. In addition, significant increase (P_{0.05}) of cuttings aged in cinnamic acid, phenol, *o*-hydroxyphenyl (Catechol) at concentrations (10⁻³, 10⁻⁵ and 10⁻⁵) M, respectively compared to control (*d*/H₂). All of these compounds caused offsetting or delaying of the oxidative processes that occurs during ageing as anti-oxidants which, acts as free radical scavengers against oxidative process products and then promoting IAA biosynthesis. Quantitative estimation of IAA by spectrophotometric method verified a highly significant increase of IAA content in hypocotyl of cuttings aged in the above compounds. These results directed the attention about the electronic conjugation area of ascorbate and hydrogen bonding of phenolic compounds and the oxidation of hydrogen – oxygen bond between hydroxyl groups of sugars in terms of rooting response of mung bean cuttings, which is influenced primarily by auxins.

However, the capability of phenolic compounds under investigation (as anti- oxidants) for trapping free radicals, may be attributed to the presence of high electronic conjugation in these compounds comparing to others. Obviously, from the structural formula of cinnamic acid, the direction of electronic conjugation is from the ring to the carboxyl group, this electronic movement depends on ionization ability of acidic hydrogen atom in the carboxyl

group. It might be a weak acid, so the electronic movement from the ring to carboxyl group was also weak too. Thereafter, the area of electronic conjugation from the ring to carboxyl group was inactive because of the weakness of hydrogen acidity of (OH) group. This leads to decline in its activity as anti-oxidant comparing to *o*-coumaric acid. Seemingly, the roots number of cuttings aged in cinnamic acid was 19.1 roots whereas, in *o*-coumaric acid, it was 42 roots.



Moreover, cutting aged in high concentrations of ascorbate (200- 500 ppm) responded as it was the case in fresh cuttings, and attributed to ascorbate as anti-oxidant having electronic conjugation system between atom No. (1) and atom No. (3) as illustrated below. And also having less Electronic conjugation area compared to phenols if characterised as anti-oxidant (three carbon atoms and three oxygen groups).

Mostly, sugars are considered as anti-oxidants through oxidation of oxygen-hydrogen bond in hydroxyl groups, although it is weakly ionizable due to the absence of electronic conjugation in the ring forms of sugars nature as compared to phenolic compounds and vitamin C. The role of sucrose as anti-oxidant resides in modifying the activity of enzymes particularly that involved in auxin-metabolism via inhibiting the activity of IAA-oxidase and hence, promoting IAA level in hypocotyl (Root initiation zone). This was confirmed by quantitative measurements of IAA in the current study in hypocotyl of aged cutting in sucrose 3%, which developed 15.411 m moles compared to control treatment 11.067 m moles.

Glutathione (GSH)

In terms of ARF, low conc. of GSH (10^{-10} M) at PH (6.4) promotes, rooting response in aged mung bean cuttings (14.5root) compared to control (11.1 roots) (Alwan, 2004). Although, statistically it is not significant but coincided with a significant increase in IAA-biosynthesis (55.2%). Meanwhile, high conc. of GSH were characterised by its inhibitory effect with necrosis in primary leaves and damage in hypocotyls (at Particularly high cons. (10^{-3} – 5×10^{-3} M).

Promotion of rooting response in mung bean cuttings were supplied with GSH, attributed to the central role of GSH in anti-oxidant defences and its capability for regeneration of another strong water soluble anti-oxidant, ascorbate via Ascorbate – GSH cycle (Noctor and Foyer, 1998).

The role of GSH as anti-oxidant was confirmed by many studies such as Foyer and Rennenberg (2000). The latter mentioned that GSH is a thiol, more available in plants and participate in heavy metals tolerance & detoxification. However, many plants have the ability to synthesis polypeptides called phytochelatins similar in their structures to GSH having an important role in detoxification of peroxides that regenerated in presence of ROS (Schmidt and Jager, 1992).

III) Enzymatic anti-oxidant defence systems

Recently (Shaheed *et al.*, 2010b) were found a decline in the activity of SOD (fig. 1) & CAT (fig. 2) (46.6% & 78.9%) respectively, accompanied by the decline of IAA content (29.6%) in aged cuttings. The above results developed the 1st physiological indicator for what was happened during ageing, due to the oxidative processes (scandlios, 1993), that caused declining of IAA into 12.25 mM in primary leaves and 8.02 mM in root initiation zone (hypocotyl) compared to (15.82 & 13.66 mM) respectively. These results were confirmed by Hartmann *et al.* (1988) in leaves during ageing or senescence.

In addition, the decline of SOD & CAT activities develops clear speculation about oxidative processes, its role in reduction the biosynthesis of pre-requisites of rooting response such as IAA.

Some minerals are capable of controlling the processes that occur during ageing completely or partially such as, FeSO_4 , CuSO_4 , ZnSO_4 and MnSO_4 at 0.1 g/L for all except MnSO_4 at 0.18 g/L. The elements of these salts have been chosen depending on their roles in the biosynthesis of certain enzymes such as SOD (Scandlios, 1993) & ascorbate oxidase (Salisbury and Ross, 2010). It is possible to interpret the role of these elements through their effects in:

Firstly, increasing the activity of anti-oxidant system: SOD in cuttings, aged in ZnSO_4 & FeSO_4 and CAT in cuttings, aged in MnSO_4 and FeSO_4 . This may confirm the ability to increase synthesising these enzymes during ageing of cuttings in such solutions. Exogenous supply of such mineral may compensate its depletion during ageing. However, Gonzalez and his collages (1998) found a pronounced increase in SOD activity when *Phaseolus vulgaris* leaves treated with Mn.

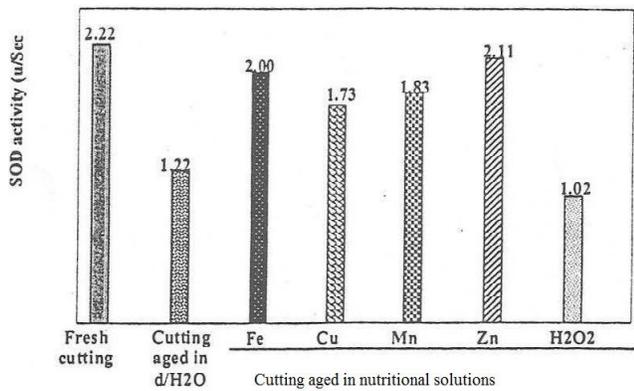


Fig. 1 : Determination of superoxide dismutase activity in fresh cuttings & cuttings aged in d/H₂O or in nutritional solutions (unit/sec.). LSD 0.05 = 0.860, LSD 0.01 = 1.192

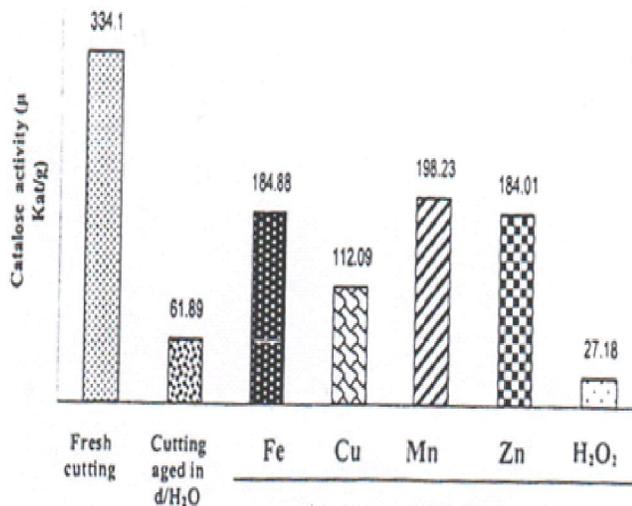


Fig. 2 : Determination of catalase activity in primary leaves of fresh cuttings & in cuttings aged in d/H₂O or in nutritional solutions (µKat/g).L.S.D. 0.05 = 60.35, 0.01 = 83.76.

Secondly, reducing of lipid peroxidation. A severe reduction in MDA approaches 50% in cuttings aged in & (1.6 & 1.4 mg/g respectively) and statistically not differ from its values in fresh cuttings. The effect of elements (Such as Fe, Zn, Cu, and Mn) in increasing the efficiency of enzymatic anti-oxidant system leads to raising rooting response of aged cuttings (table 4).

Thirdly, increasing IAA content increased in all parts of cuttings aged in the above mineral solutions such increase was interpreted via the role of these solutions in the biosynthesis of IAA *per se*, Particularly Zn (Salisbury and Ross, 2010) by activation of some enzymes in IAA biosynthetic -pathway. However, IAA was declined in cuttings aged for 3-days in d/H₂O, such cuttings were already grown in Hoagland solution lacking for Zn, Cu, Fe, Cu (Shaheed *et al.*, 2009b).

i) Attempts to delay ageing

Attempts for controlling the degenerative processes that occur during ageing of plant tissues were aimed to delay or overcome partially or completely of these processes.

The previous attempts were represented in different mechanical or physical – chemical means such as, removal of flowers or fruits to delay the onset of ageing via lowering the competition on nutritional substances between young developing & old organs (Klubertanz *et al.*, 1996), Pruning for developing new juvenile branches or exogenous supply of nutrients (Hartmaun *et al.*, 1990). In addition, the chemical treatment of *Centranthus ruber* leaf strips with Ceralenin and CaSO₄ leads to repair membrane and maintaining permeability (Abraham and Reinold, 1980).

Recently, there are numerous physico-chemical attempts has been done to delay ageing in mung bean cuttings. Some are failed, some succeeded partially & others succeeded completely.

The 1st kind of attempts that failed to delay ageing involved employment of the followings :

- Polyamines (*e.g.* Spermine) at 10⁻²-10⁻⁷ M (Shaheed, 1987).
- Boron at 10 µg/ml (Shaheed, 1987).
- Excising of hypocotyl base of mung bean cutting (Shaheed, 1987).
- Alkaloids (*e.g.* Harmalol, 8-Hydroxy-Quinoline and Nicotine) all at 10⁻¹¹-10⁻³M (Shaheed, 1997).
- KCl and NaCl at optimal conc. for rooting response of fresh cuttings (0.08 & 0.02M), respectively (Shaheed and Salim, 2002a).
- Aqueous extract of Liquorice (*Glycyrrhiza glabra*) roots at (0.1-10%) (Shaheed and Al-always, 2001).
- Aqueous extract of Thyme (*Thymus vulgaris*) shoots of (0.1-10%) (Abu-altimen, 2003)
- Aqueous solutions trapping volatile oils of Myrtle (*Myrtus communis*) leaves (Shaheed *et al.*, 2009a).

The 2nd kind of attempts that succeeded partially in delaying ageing involved the employment of:

- Dilute solutions of IAA & IBA at 10⁻⁹-10⁻¹⁰M (Shaheed, 1987).
- Keeping cutting in darkness during the ageing period (Shaheed, 1987).
- The interaction between boron, auxin, and

Table 4 : Effect of nutritional solutions supplied during ageing on rooting response of mung bean cuttings.

Treatments	Subsequent treatment for 24h in	Mean root number/cutting
Fresh cuttings	d/H ₂ O	4.88
Fresh cuttings	IAA, 10 ⁻⁴ M	56.33
Cuttings aged in Cl/H ₂ O	d/H ₂ O	5.81
Cuttings aged in d/H ₂ O	IAA, 10 ⁻⁴ M	25.0
Cuttings aged in FeSO ₄ (0.18) g/l	IAA, 10 ⁻⁴ M	19.83
Cuttings aged in FeSO ₄ (0.1) g/l	IAA, 10 ⁻⁴ M	40.5 *
Cuttings aged in FeSO ₄ (0.02) g/l	IAA, 10 ⁻⁴ M	24.17
Cuttings aged in CuSO ₄ (0.18) g/l	IAA, 10 ⁻⁴ M	21.33
Cuttings aged in CuSO ₄ (0.1) g/l	IAA, 10 ⁻⁴ M	39.5 *
Cuttings aged in CuSO ₄ (0.02) g/l	IAA, 10 ⁻⁴ M	22.5
Cuttings aged in ZnSO ₄ (0.18) g/l	IAA, 10 ⁻⁴ M	11.33
Cuttings aged in ZnSO ₄ (0.1) g/l	IAA, 10 ⁻⁴ M	45.5 **
Cuttings aged in ZnSO ₄ (0.02) g/l	IAA, 10 ⁻⁴ M	18.17
Cuttings aged in MnSO ₄ (0.18) g/l	IAA, 10 ⁻⁴ M	40.33 *

L.S.D. 0.05 =14.48, L.S.D. 0.01=17.57 **complete inhibition *partial inhibition

excising of hypocotyl base (3mm) (Shaheed, 1987).

- d) Aqueous extracts of Garden lettuce (*Lactuca sativa*) seeds, Fenugreek (*Trigonella foenum – graecum*) shoots and chamomile (*Anthemis nobilis*) flowers at 10%, 0.1% and 1% respectively (Shaheed & Al-alwani, 2001).
- e) Ammonium sulphate at 0.025% (Shaheed and Abu – altimen, 2009).
- f) Glutathione at 10⁻¹⁰M (Alwan, 2004).
- g) Cinnamic acid at 10⁻³M and Phenol at 10⁻⁵M in absence of supplied auxin (Shaheed *et al.*, 2010a).

The 3rd kind of attempts that succeeded completely in delaying (overcoming 100%) ageing processes involved the employment of:

- a) Cinnamic acid at 10⁻³M in presence of auxin (Shaheed, 1997).
- b) Aqueous extracts of Fenugreek, Anise (*Pimpinella anism*), Milfoil (*Achillea sentoonlina*), Negella (*Nigella sativa*) all at 1% and common nettle (*Urtica dioica*) at 0.1% (Shaheed & Al-alwani, 2001).
- c) Aqueous extracts of Ginger (*Zingiber officinale* Roscoe) rhizomes and passion flower (*Passiflora incarnate*) leaves at 0.01% for both (Abu-altimen, 2003).
- d) Sucrose at 1.5%, Ascorbate at 400 mg/L and glucose at 1% and 1.5% (Shaheed and Abu-altimen, 2009).

e) Phenolic compounds (e.g. *o*-coumaric acid, caffeic acid and *P*. hydroxy – Quinone) at 10⁻³M for all and Ascorbate at (200–500) ppm and sucrose at 3% (Shaheed *et al.*, 2010).

f) Cysteine, 10⁻³M, Methionine, 10⁻⁴M, Salicylic acid, 10⁻⁴M and citric acid, 10⁻⁹M and combination between them (Kadhun, 2011).

Obviously, spermine was without effect on the diminishing rooting response resulting from ageing. However, the time of spermine application might, of course, be crucial inasmuch as it might disturb the balance of polyamines and growth regulators. Indeed, Lin (1984) suggested that the early action of natural growth regulators is not on polyamine metabolism but, rather, that the levels of polyamines may be crucial for the regulation of hormone action. The similarity between the fluctuation in polyamine biosynthesis in relation to the distinct phases of adventitious root formation, as indicated by Friedman *et al.* (1985), and the concentration of auxin in relation to its action through the same phases (Norcini *et al.*, 1985) are consistent with this idea. So too is the similarity between the roles of polyamines and boron in cell division and growth (Bagni *et al.*, 1981).

Whilst exogenous boron, essential for the development of root primordia and growth (Middleton *et al.*, 1978b), may be supplied during growth of stock seedlings, it is not required until between 24 and 48 after the initial 24 h supply of exogenous auxin to cuttings (Shaheed, 1987). It has been suggested that the rapid ageing of stem cuttings in the light, and the associated lower rooting response to high concentrations of exogenous auxin, is due to a

deficiency of boron (Middleton *et al.*, 1980). This is clearly not the case in view of the data presented by (Shaheed, 1987). Boron, whether supplied during growth of the stock seedlings or during the ageing period itself, had no influence on the ageing processes, which lead to diminished rooting. Such ageing in the light was, however, clearly delayed or partially overcome by the supply of low levels of auxin, which in themselves do not stimulate rooting (Jarvis *et al.*, 1983). From the work presented here, it is clear that enhanced rooting results from an immediate application of auxin as soon as cutting are made. If auxin was supplied at a high concentration, then rooting was initiated, but if the level was low, no initiation occurred, although responsiveness to a subsequent high dosage of auxin was retained. It is not immediately obvious why this should be so, but it could be an example of the influence of supplied auxin in enhancing auxin – binding activity, as suggested by Trewavas (1980).

Alternatively, there may be a continual need to maintain a crucial level of auxin somewhere in the cutting. If this level is not maintained by exogenous supply, then ‘ageing’, as evidenced by a diminishing capacity for root formation, ensues.

The possibility of darkness and etiolation enhancing auxin accumulation at the base of cuttings, as well as the limited supportive data, has been discussed by several authors (*e.g.* Kawase, 1965; Eliasson, 1978 and 1980). Given the possible interaction between the transport of auxin into the hypocotyl and photodestruction of auxin in root regeneration.

Despite being essential for root primordium development and subsequent growth, boron can be antagonistic to the effects of auxin, at least when the latter is presented at low concentration.

Collectively, these observations are consistent with reduced levels of auxin in the hypocotyl, furthermore, boron has been shown to enhance the activity of IAA oxidase (Parish, 1968).

Excision of the base of the hypocotyl at the end of ageing period reduced the number of roots induced by IBA in cuttings aged in water, whereas, excision significantly enhanced rooting in those aged in borate. Possibly, because uptake of IBA is limited and boron is adequate to facilitate whatever potential basipetal transport of auxin arises. It took due note of the observation of Tang and dela Fuente (1986a, b) that boron, as well as calcium, is necessary for auxin transport. In other cuttings, which are not deficient in borate, enhancement of rooting due to excision treatments has been reported (*e.g.* Eliasson, 1981; White and Lovell, 1984).

It should be noted that the suggestions above distinguish between the role of boron when the latter is supplied immediately cutting are made, and that of boron which is necessary 72h and thereafter, when root primordia develop and grow.

j) Chemical potentiation of Aqueous plant extracts

Shaheed and Abu – altimen (2009) has been studied for the 1st time the chemical potentiation of aqueous extract of Ginger (*Zingiber officinale* Roscoe) rhizome in terms of ARF of mung bean cutting, by adding chemicals of different nature such as Carbohydrates (*e.g.* sucrose & glucose), vitamins (*e.g.* ascorbic acid), and Nitrogenous compounds (*e.g.* $(\text{NH}_4)_2\text{SO}_4$). However, sucrose, glucose, and ascorbic acid at conc. 1.5%, 1-1.5% and 400 ppm respectively were delaying ageing processes and making the aged cuttings capable of responding as it was the case in fresh cuttings.

On the other hand, potentiation of ginger extract in terms of ARF was succeeded by adding $(\text{NH}_4)_2\text{SO}_4$ (0.025%) and glucose (1-2%). Surprisingly, rooting response was not only raised to the level of that in fresh cuttings but, markedly raised beyond that of fresh cuttings. Sucrose was without effect on potentiation of ginger extract, perhaps because of restriction the active substances or acts as an antagonist for the activity of some beneficial sugars, which already present in the extract (Haissig, 1986). However, the obvious role of glucose in potentiation of ginger extract may be attributed to its binding to (-OH) in the phenyl group of the 6 – Gingerdiol and forming glucosidic compound. The latter has anti – oxidant activity equivalent to that present in Gingerdiol and Gingerol. Those glucosides act as intermediates for 6- Gingerdiol and hence, improving the functional properties of ginger extract (Sekiwa *et al.*, 2000).

Ascorbic acid has no synergistic effects with ginger extract constituents. Possibly, the reason may reside, to the binding of ascorbate into secondary compounds in the extracts (Sharma *et al.*, 2000), such as tannin (qualitative assay) as anti – oxidant. Phenolics and tannin are promotive substances for rooting response were present in the extracts, it might bind to ascorbate. So the suggestion might be excepted if ascorbate conc. increased more than 400 mg/L in order to potentiate the extract (Shaheed *et al.*, 2009a).

Consequently, the optimal conc. of $(\text{NH}_4)_2\text{SO}_4$ (0.025%) was increased the potentiation of Ginger extract in terms of ARF. However the roots no. is (122) which is much more compared to crude extract (70.2 roots) or cuttings were treated with the $(\text{NH}_4)_2\text{SO}_4$ alone (77.4

roots). This might be associated with new C/N ratio that formed in extract after supplying $(\text{NH}_4)_2\text{SO}_4$, which may represent ideal ratio for rooting.

The best rooting response occurred at high C/N (Haissig, 1986). While the C/N ratio in Ginger extract is 68.2/ 8.3 represented by carbohydrate/protein (So any addition of $(\text{NH}_4)_2\text{SO}_4$ to the extract will raise the level of Nitrogen thereafter, making the C/N ratio in a delicate balance, better for rooting. keeping in mind, supplying of $(\text{NH}_4)_2\text{SO}_4$ increase the Alkalinity of extract (Hartmann *et al.*, 1990) to the optimal value for enzymes that present in extract particularly, alcohol dehydrogenase (ADH), which acts on oxidation of Geranial to Geranial that is responsible for the improvement of physiological properties of Ginger rhizome extract (Sekiwa *et al.*, 2000).

Conclusion

The 1st four of ageing hypotheses has been verified, some in at least two different approaches, as well as new hypothesis (reversed Na^+/K^+ pump action hypothesis) was suggested. In addition, to overcoming (controlling) ageing processes by employing different physico-chemical means mostly achieved by using medicinal herb extracts.

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