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Impairment of Scavenging Enzymes mediated Seed Ageing in Jamun (*Syzygium cuminii*) seeds

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Abstract: **The present study was carried out to elucidate the mechanism of seed deterioration in ageing Jamun seeds. The naturally dried (Jamun) seeds with the decline in water content show a corresponding decline in % germination. The damage caused by water removal are due to the production of reactive oxygen species which when produced in large amount are the source of damage to membrane lipids, proteins and nucleicacids. As a consequence, dessication tolerance and prolonged longevity in the dessicated state depend on the ability to scavenge the reactive oxygen species, by using scavenging enzymes. Battery of scavenging enzymessuch as Superoxide dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) and Guaiacol peroxidase (GPX) are playing vital role in protecting ROS induced cellular damage. The fresh Jamun seeds exhibiting absolute germination recorded highest level of of scavenging enzymes SOD, CAT and APX both in the axis and cotyledons that were reduced gradually with decline in percent germination in response to ageing (slow drying). The decrease in germinability of Jamun seeds was correlated with the increased accumulation of total peroxides due to decrease in the activities of scavenging enzymes SOD, CAT and APX.**

Keywords: **Reactive Oxygen Species, Antioxidant Enzymes, Superoxide dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX).**

1. INTRODUCTION

Seeds of *Syzygium cuminii* are categorized as true recalcitrant as they are shed with high water content (0.93 g H_2O g^{-1} DM).The freshly plucked seeds exhibit 100 % germination but a fast loss of germinability (within a short period of 30 days during storage under natural condition) was discernible as the seed dessicated from 0.93 g H_2O g^{-1} DM to 0.23 g H_2O g^{-1} DM water content. Drying of these seeds below critical moisture content (below 47.71% moisture content) leads to imbalance in the levels of ROS e.g. of superoxide anion radical ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) which may initiate oxidative stress and thus contribute to viability loss during ageing [1],[2],[3],[4]. ROS are acknowledged as the main factors causing various damages in cells by means of oxidation, as it leads to cell death. The scavenging of free radicals and prevention of potential molecular damages are controlled by the defense mechanisms activated in plant cells. Those mechanisms involve low molecular and enzymatic antioxidants[5],[6],[7],[8].Antioxidant enzymes such as Superoxide dismutase (SOD), Ascorbate peroxidase (APX) and Catalase (CAT) are considered to be the main protective compounds engaged in the removal of free radicals and activated oxygen species[9],[10]. Reduced cellular and membrane damage has been linked to increased enzymatic defence against toxic ROS [11],[12] in sunflower[13] seeds.

The axis and cotyledon of seed exhibited differential expression of antioxidant enzymes quantitatively and qualitatively. Comparatively higher level of SOD and CAT activities were recorded in the axis than the cotyledons of dry seeds of *Azadirachta indica* [14]. Differential magnitude of scavenging enzymes in the axis and cotyledon of dry seeds may be due to different extent of hydration level of these tissues (Wojtyla et al.,2006). The objective of this research was to follow changes in the activity of SOD, CAT and APX in ageing Jamun seed for unraveling the role of these scavenging enzymes in the maintenance of seed viability in desiccating sensitive seeds. Further activities of these enzymes were monitored separately in axis and cotyledon of dehydrating Jamun seeds.

2. MATERIALS AND METHODS

2.1 Site of Fruit Collection

Jamun (*Syzygium cuminii* L.) fruits were collected from avenue trees near by Bhilai (Chhattisgarh), India. Nearly 80 plus trees were marked for collection of fruits. Seeds were collected for three consecutive years.

2.2 Fruit collection

Freshly mature fruits of Jamun were plucked manually during June-July. The collected fruits were transported to the laboratory within one hour of collection. The healthy fruits were sorted out. The fruit is a drupe, variable in size, oblong or sub-globose, crowned with a persistent truncated first pink, then black with pink juicy mesocarp.

2.3 Seed extraction

Seeds were extracted by rubbing the fruit with sand to remove pulp. Three replicates of 50 seeds each were used for determination of moisture content. Remaining pulp free seeds were then washed thoroughly with tap water to remove traces of pulp and allowed to air-dry to initial moisture contents. Each fruit contains one seed which is 1-2 cm long, oblong in shape green or brown in colour.

2.4 Seed drying and storage

The mature Jamun seeds collected were for conducting experiments related to slow (natural drying at laboratory conditions RH 30% and Temperature (27-30^{o}C). The seeds was subjected to slow drying by spreading them in one layer in a perforated basket at ambient conditions (27-30^{o}C at RH 30%).

2.5 Moisture content and estimation of water content

Moisture contents of slow dried seeds were determined by oven drying the seeds for 72 hours at 103°C [15]. Five replicates with 10 seeds each were used to determine the moisture content, on a fresh weight basis. The seed moisture content was determined by the following formula and was expressed in percentage:

$$\% \text{ Moisture Content} = \frac{\text{Seed Fresh Weight - Seed Dry Weight}}{\text{Seed Fresh Weight}} \times 100$$

Water content of slow dried seeds was determined by the method given by [16]

$$\text{Water Content} = \frac{\text{Seed Fresh Weight - Seed Dry Weight}}{\text{Seed Dry Weight}} \text{ x g } H_2O \text{ } g^{-1}DM$$

2.6 *Enzyme Extraction*

Enzyme protein was extracted by homogenizing 500 mg of frozen axis/cotyledons of the jamun seeds, dehydrated for various intervals, in 2 ml borate buffer ((0.2 M, pH 7.4) and the clear supernatant was collected after centrifugation at 10,000 rpm for 20 minutes. The supernatant was used as a source of enzyme for measuring Superoxide dismutase (SOD), Catalase (CAT) and Ascorbate Peroxidase (APX).

a) Superoxide Dismutase (SOD) Activity

The method followed for determining SOD activity was given by [17]. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the pyrogallol auto-oxidation monitored at 420 nm. Auto-oxidation of pyrogallol was monitored at 420 nm by recording the kinetics of the reaction mixture containing 2.74 ml of 50 mM Tris-HCl buffer (BDH), (pH 8.2), 1 mM DETAPAC (Sigma) and 60 µl pyrogallol that were prepared in 10 mM HCl. For SOD assay, 0.2 ml of enzyme extract was added to 2.74 ml of Tris-HCl buffer and the absorbance was adjusted to zero.

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The reaction was initiated by adding 60 µl of pyrogallol and change in absorbance was recorded at 420 nm. Enzyme activity was calculated and expressed on the basis of soluble protein as units of SOD unit min^{-1} g^{-1} FW.

b) Catalase (CAT) Activity

CAT activity was determined by monitoring the decomposition of H_2O_2. The method was given by [18]. Enzyme activity was recorded by taking, in a glass cuvette 2.68 ml of potassium-phosphate buffer (37.5 mM, pH 6.8), to which 120 µl enzyme extract was added and the absorbance at 240 nm was zeroed. Catalase activity was triggered by adding 200 µl of H_2O_2 (60 mM) (BDH, India). The change in absorbance was recorded for 2 minutes at 15 seconds interval. Absorbance was recorded at 240 nm. Unit of Catalase activity was expressed as A_{240} min^{-1} mg^{-1} protein.

c) AscorbatePeroxidase (APX) Activity

APX assay was performed according to [19]. The rate of hydrogen peroxide dependent oxidation of ascorbate, as an electron donor, was determined in a reaction mixture that contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM ascorbic acid and enzyme, in a total volume of 1 ml. The reaction was started by addition of hydrogen peroxide. The oxidation rate of ascorbate was estimated by monitoring the decrease in absorbance at 290 nm after the start of the reaction. Unit of Ascorbate Peroxidase activity was expressed as A_{290} min^{-1} μg^{-1} protein.

The activities of SOD, CAT and APX were determined in enzyme extract of axis and cotyledon of jamun seeds separately. The results corresponded to the means of the values obtained with three replications.

3. RESULTS

3.1 Desiccation and Loss of Viability

Absolute germination (100%) was recorded in Jamun seeds during slow drying of seed water content from 0.93 to 0.86 g H_2O g^{-1} DM. Thereafter, with quick decline in water content, a corresponding decline in %germination was also registered, i.e. 95% germination was recorded at water content 0.81 g H_2O g^{-1} DM and 80% at water content 0.42 g H_2O g^{-1} DM. Complete loss of germination was noticed at the time when seed was desiccated to 0.23 g H_2O g^{-1} DM. Along with the per cent germination, the germination index of the seed also decreased considerably with loss of water content during seed storage.

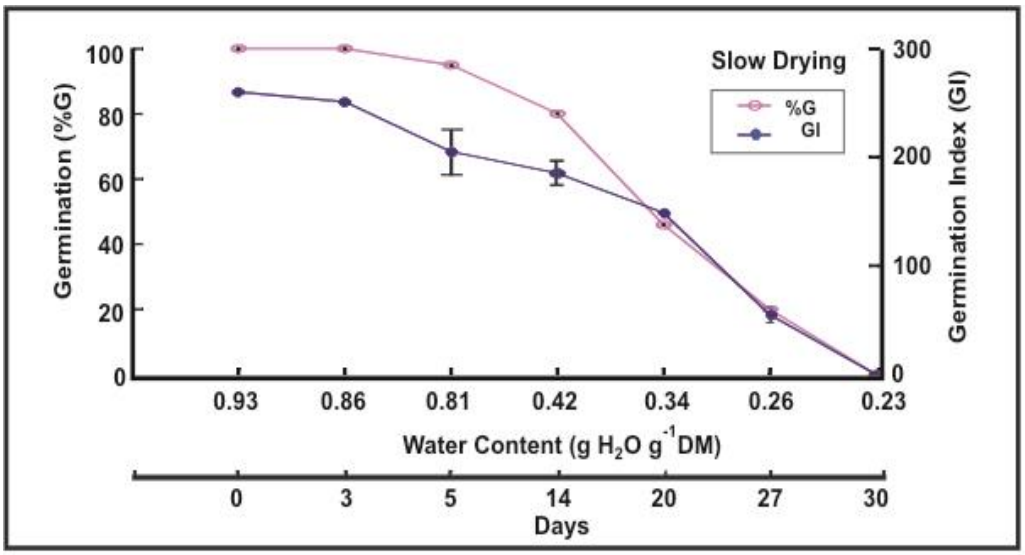

Figure 1 The decline in percentage of germination and germination index of *Syzygium cuminii* seeds during storage (slow drying) under ambient conditions. Correlation between water content of dehydrating seed and percent germination of seed was r = 0.90, whereas with germination index was r = 0.89, while the correlation between period of slow drying and percent germination of seed was r = - 0.97 and with germination index was r = - 0.96. Data are mean of 5 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol

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3.2. Antioxidant Enzymes

a) Superoxide Dismutase (SOD)

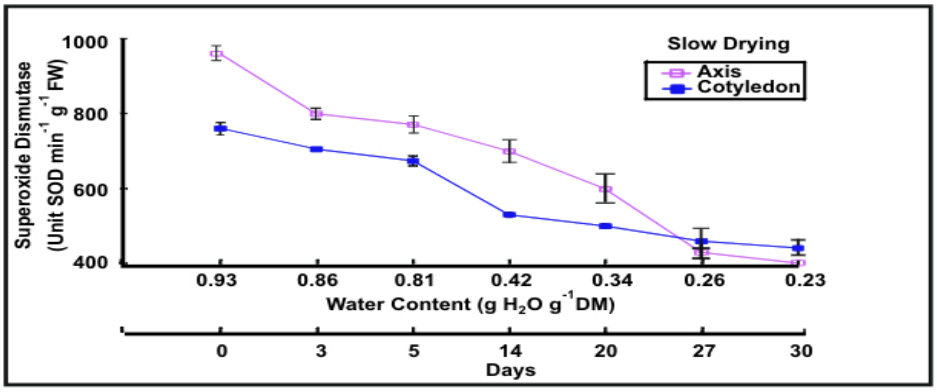

Figure 2 Decline in the superoxide dismutase activity in embryonic axis and cotyledon of *Syzygium cuminii* seeds with decline in water content during slow drying. Correlation between superoxide dismutase activity and loss in water content of dehydrating seed was r = 0.93 (axis) and r = 0.99 (cotyledon) and correlation between superoxide dismutase activity and days of slow drying was r = - 0.98 (axis) and r = - 0.97 (cotyledon). Data are mean of 3 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

Significantly higher levels of SOD were estimated both in the axis and cotyledon of fresh jamun seeds. Dehydration of seeds resulted in the gradual loss of SOD measured in the axis and cotyledon. For example, the SOD activity that was 960.78 and 760.72 unit SOD min^{-1} g^{-1} FW respectively in the axis and cotyledon of undesiccated seeds decreased almost to 50% both in the axis (401.05 unit SOD min^{-1} g^{-1} FW) and cotyledon (440.61 unit SOD min^{-1} g^{-1} FW) as the seeds dehydrated during slow drying to maximum i.e. 0.23 g H_2O g^{-1} DM. Although the SOD activity was higher in the axis than the cotyledon the ratio of loss of SOD activity at various stages of seed dehydration was similar. SOD activity was positively correlated with the loss of water content in the axis (r = 0.93) and cotyledon (r = 0.99) of jamun seeds during slow drying, while a negative correlation was established between SOD activity in the axis (r = - 0.98) and cotyledon (r = - 0.97) with the days of slow drying.

b) Catalase (CAT)

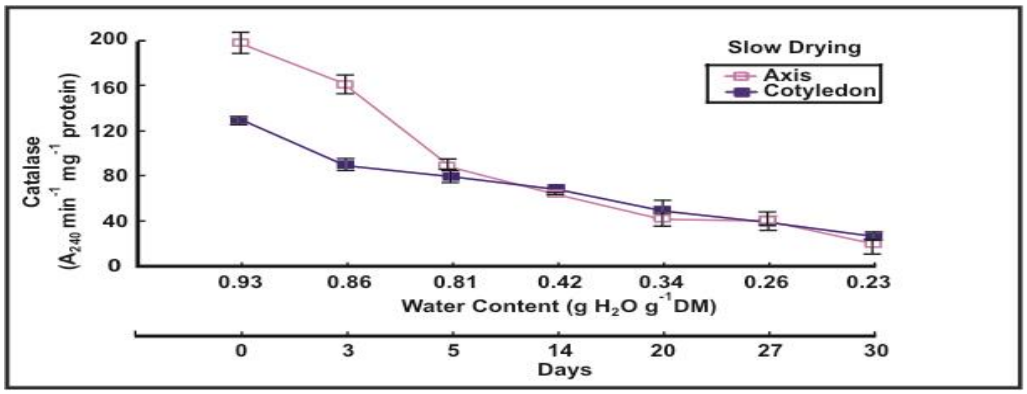

Figure 3 Decline in the catalase activity in embryonic axis and cotyledon of *Syzygium cuminii* seeds with decline in water content during slow drying. Correlation between catalase activity and water content of dehydrating seed was r = 0.92 in both axis and cotyledon, whereas correlation between catalase activity and days of slow drying was r = - 0.90 (axis) and r = - 0.94 (cotyledon). Data are mean of 3 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

The pattern of CAT activity measured in the axis and cotyledon was almost similar to SOD activity. Catalase activity declined gradually from 198.48 to 88.47 A_{240} min^{-1} mg^{-1} protein, almost 2-fold, in the embryonic axis when the fresh seeds with water content 0.93 were dehydrated to 0.81 g H_2O g^{-1} DM. Later, the dehydration of seeds from 0.81 to 0.23 g H_2O g^{-}

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[1] DM during slow drying resulted in very sharp loss in the CAT activity in axis i.e. 88.47 to 19.94 A_{240} min^{-1} mg^{-1} protein, almost 4-fold. Similar pattern of CAT activity was discernible in the cotyledon, although with relatively less magnitude, at least in the seeds dehydrated from 0.93 to 0.81 g H_2O g^{-1} DM. Cotyledonary tissue exhibited highest CAT activity i.e. 129.19 A_{240} min^{-1} mg^{-1} protein in the fresh seeds. The CAT activity decreased from 129.19 to 79.53 A_{240} min^{-1} mg^{-1} proteins in the cotyledon in response to desiccation of fresh seeds to 0.81 g H_2O g^{-1} DM. The levels of CAT activity were similar to that of axis in the later stages of dehydration of seeds during slow drying. CAT activity was positively correlated with the loss of water content in the axis (r = 0.92) and cotyledon (r = 0.92) of jamun seeds during slow drying, and a negative correlation was established between catalase activity in the axis (r = - 0.90) and cotyledon (r = - 0.94) with the days of slow drying.

c) ***Ascorbate Peroxidase (APX)***

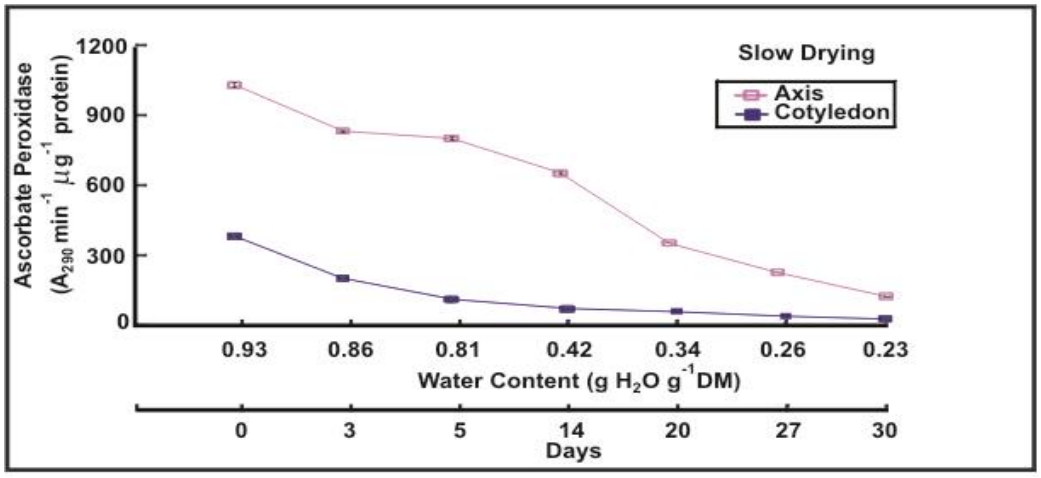

Figure 4 Decline in the ascorbate peroxidase activity in embryonic axis and cotyledon of *Syzygium cuminii* seeds with decline in water content during slow drying. Correlation between ascorbate peroxidase activity and water content of dehydrating seed was r = 0.95 (axis) and r = 0.86 (cotyledon) whereas correlation between ascorbate peroxidase activity and days of slow drying was r = - 0.99 (axis) and r = - 0.84 (cotyledon). Data are mean of 3 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

Magnitude of dehydration-induced changes in APX activities was exceptionally pronounced in the axis than in the cotyledon of jamun seeds. For example, the APX level in the axis of fresh seed was 1030.72 A_{290} min^{-1} μg^{-1} protein whereas it was 380.41 A_{290} min^{-1} μg^{-1} protein in the cotyledon. Dehydration of seeds from 0.93 to 0.23 g H_2O g^{-1} DM was associated with rapid loss in APX activity from 1030.72 to 120.72 A_{290} min^{-1} μg^{-1} protein, almost 8.5-fold loss in axis whereas in cotyledon it was from 380.41 to 10.66 A_{290} min^{-1} μg^{-1} protein, more than 35-fold loss. Loss of APX activity was proportionately marginal (from 1030.72 to 640.18 A_{290} min^{-1} μg^{-1} protein) in axis during dehydration of seeds from 0.93 to 0.42 g H_2O g^{-1} DM but further dehydration to 0.23 g H_2O g^{-1} DM exhibited sharp loss to 120.72 A_{290} min^{-1} μg^{-1} protein. In general, the APX activities were significantly higher in axis than in the cotyledon. The APX activity in axis (r = 0.95) and cotyledon (r = 0.86) was positively correlated with the dehydration of seeds, and a negative correlation was established between APX activity in the axis (r = - 0.99) and cotyledon (r = - 0.84) with the days of slow drying.

4. DISCUSSION

The fresh jamun seeds exhibiting absolute percent germination recorded highest levels of SOD, CAT and APX, both in axis and cotyledons that were reduced gradually with decline in percent germination in response to ageing (slow drying). It appears that massive reduction in the antioxidative activity (SOD, CAT and APX) permitted excessive accumulation of AOS which was responsible for non-viability in Jamun seeds. Damaging amounts of AOS as a result of impaired activities of detoxifying enzymes in seeds of *Shorea robusta* [20],[21], *Helianthus annus* [22],[23],[24] *Vigna radiata* [25] *Azadirachta indica* [14], *Gossypium hirsutum* [26] and *Fagus sylvatica* [27] was linked to rapid loss of viability in desiccation sensitive seeds dried below critical moisture content. Comparatively, higher antioxidant capacity rendered by higher levels of antioxidant enzymes in the viable seeds than in the non-viable seeds is widely acknowledged and

correlated with higher seed viability and vigour [28]. It is argued that higher levels of antioxidant enzymes protect the seed viability from AOS induced damage owing to oxidation/peroxidation of lipid, protein and DNA that are predominantly contributing to dehydration or ageing induced deterioration. In Jamun seeds, we noted the decrease in the activity of superoxide dismutase (Fig 2), Catalase (Fig 3) and Ascorbate Peroxidase(Fig 4) in parallel with the slow drying (ageing).

SOD is considered as a key enzyme in the regulation of intracellular concentration of superoxide radical and peroxides. This removes superoxide and hence increase the risk of hydroxyl radical formation from superoxide via the metal catalyzed Haber-Weiss type reaction [29]. In the present study SOD activity decreased with ageing which may be responsible for the increase of AOS (hydroxyl radical) with ageing.

The H_2O_2 is reduced to water by CAT in mitochondria whereas in cytosol H_2O_2 is detoxified by APX [30]. Faster rate of loss of CAT both in the cotyledon and axis during slow drying perhaps, permit accumulation of higher amounts of H_2O_2 that may be considered important in causing faster loss of viability during ageing. Reduced catalase activity has been attached with loss of vigour and viability owing to reduced capacity of detoxification of H_2O_2 in the early steps of seed imbibition leading to lipid peroxidation in sunflower seed [31].

Relatively higher levels of APX were reported in recalcitrant seeds compared to orthodox seeds [32]. As the recalcitrant seeds enter into germination phase from developmental phase without sufficient lag period (as in orthodox seeds) due to abbreviated maturation phase during development, the presence of higher APX is essential for maintaining critical levels of H_2O_2 required during germination. The ageing of Jamun seed leads to exceedingly faster rate of loss of APX activity both in the axis and cotyledon.. The rate of loss of APX activity in response to ageing was relatively far higher in cotyledons than the axis. Exceptionally greater loss of APX activity in the cotyledon of slow dried seeds indicated significant role of cotyledon in contributing the quality of recalcitrant seeds.

The pattern of oxygen radical processing enzymes; SOD, CAT and APX estimated in the axis and cotyledon of dehydrating jamun seeds clearly revealed vital participation of these enzymes in conferring desiccation tolerance and maintaining higher seed viability and vigour in Jamun seeds with water content above CWC. Drying jamun seeds below critical water content invoked disturbance in metabolic balance of recalcitrant seeds both in axis and cotyledon that caused over accumulation of active oxygen species (AOS), such as superoxide ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) [33] along with decline in antioxidant enzyme activities. Our data also confirmed vital participation of antioxidative enzymes in axis as well as in cotyledon of dehydrating jamun seeds in maintaining the quality of seeds i.e. high vigour and viability.

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