Identification of Abiotic and Biotic Factors Causing Deterioration During Storage and Development of Storage Techniques for Mahua (*Madhuca indica* Syn. *Bassia latifolia*) flowers

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Summary

Mahua (*Madhuca indica* syn. *Bassia latifolia*) flowers, occupy an important position in the life of the tribal in many parts of India. Particularly, the flowers of the plant are sugar rich and in certain cases it is the only source of livelihood for those people. However, its nutrient quality deteriorates during the postharvest storage and thus, poses a serious problem of adequate storage. In order to determine the cause of spoilage and to develop the measures to check it, collected flowers were stored using two methods in this investigation; first, under normally practiced conditions (NPS), i.e., the practice adopted by the flower's collectors, and second, oven dried, powdered with liquid nitrogen, and stored at 0^0C ($\pm1^0$C) in different small airtight sterilized vials labelled as laboratory processed samples (LPS). Both LPS and NPS were stored for a year i.e. from one harvesting season to the next. Experiments were carried out, at every month interval, to identify the factors responsible for spoilage of flowers during storage. LPS did not exhibit deterioration in the nutrient value throughout the year of storage, but NPS showed spoilage due to various biotic and abiotic factors comprised of moisture, temperature and microorganisms. To check postharvest spoilage various innovative storage techniques like physical and chemical treatments were experimented. Results revealed that storage at low temperature by liquid nitrogen treatment and chemical conversion to oxalic acid were the most effective techniques for a long-term storage.

Key words

postharvest storage, nutrient spoilage, abiotic factors, biotic factors, physical treatments, chemical treatments

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Introduction

Spoilage during postharvest storage is a major limiting factor for many plants and their products. It directly influences not only food availability but also economic conditions of denizens of many nations. Mahua (*Madhuca indica* syn. *Bassia latifolia*) is one of the economic plants that naturally grow in the forests of India (Anonymous, 1995). Flowers of the plant are edible and have great nutrient value; especially for its sugar richness. Majority of the tribal population who comprise more than 23 per cent of total population of Orissa (a state under the Republic of India) solely depends on this plant product for their livelihood. The flowers are also used in preparation of distilled liquor, portable spirits, vinegar and feed for livestock (Adhikary and Adhikary, 1989). The flowers show anti bacterial activity against *E. coli* and also are used against rice pest diseases (Sujatha and Das, 1988). Next to sugarcane molasses, Mahua flowers contribute as most important raw material for alcohol production (Singh and Agrawal, 1989). Usually flowering of Mahua takes place, once in a year, i.e. during March-April. When the matured flowers fall on the ground, it is collected and dried under the sun for 2-3 days, packaged in gunny bags and stored in normal environment. In every fortnight interval, the bags were opened and flowers were spread on the neatly washed ground under the direct sunlight for its drying. This is a traditional practice. However, a few months after harvesting, flowers lose its lustre and become bitter in taste as the precious corollas cannot retain its food value for long duration due to many extrinsic and intrinsic factors. It is also reported that the nutritional content deteriorated quickly just after storage of this flower for a year. So, a major part of the collected flowers become unsuitable for food and flowers' economic potential is simply wasted. Mostly the sugar content decreased upto 70% within a year of storage along with deterioration of other essential nutrients (Das et al., 2010). Although flowers contain high amount of sugar besides many other important nutrients, no systematic research work has been done so far on this aspect. So, the aim was to know the exact cause of spoilage and to develop measures to store the flowers for a longer period.

Materials and methods

Plant materials and experimental designs

Sampling, preservation and maintenance of Mahua flowers

Fresh Mahua flowers collected during March-April from different places of the state Orissa, India, were divided into two parts. One part was oven dried (ISTA, 1985) and powdered with the treatment of liquid nitrogen. The whole process was carried out inside a culture room with a temperature of approximately 10^0C. This powdered mass was transferred to different small airtight sterilized vials and stored at 0^0C ($\pm1^0$C) for future use. This was termed as laboratory processed sample, LPS. The other part was sun dried, packaged in gunny bags and stored in the normal environmental conditions. Since this method of preservation is followed by the collectors and the traders of the flowers, hence, it was termed as normally practised samples, NPS. In every fortnight interval, NPS were opened, spread uniformly under the bright sunlight for five hours (sunny days) on pre-cleaned concrete floor, so as to simulate the traditional pattern of storage. Just prior to the start of investigation, the moisture content and atmospheric temperature were recorded. Simultaneously, vials containing LPS were also checked out as a matter of routine maintenance of the samples.

Estimation of carbohydrate Content

The carbohydrate content was analyzed at 30 days interval in triplicates in both the LPS and NPS parts separately for a year. Simultaneously, carbohydrate content at highest and lowest temperature along with humidity was also investigated and emphasized.

Amounts of 1 g of both NPS and LPS were taken out separately. The samples were thoroughly crushed with 80% alcohol, transferred to the test tubes separately and incubated at 60 ^{0}C in a water bath for 15 minutes. After cooling, the samples were centrifuged for 10 minutes at 5000 rpm and the supernatants were decanted into separate test tubes. For the residue part, 80% alcohol was added and again mixed thoroughly. It was then incubated at 60 ^{0}C in water bath for 15 minutes and then cooled and centrifuged for 10 minutes at 5000 rpm. The supernatant thus obtained was added to the tubes previously kept for this purpose and the process was repeated three to four times in order to extract the soluble sugar content. The supernatants of the samples obtained were used for spectrophotometric estimation of carbohydrate content (Das *et al*., 2010).The assay mixtures in the test tubes comprised of 2 ml of Anthrone in Conc. H_2SO_4 and 1 ml of sugar solution. These test tubes containing the assay mixtures were chilled, thoroughly mixed and then heated in a water bath for 16 minutes at 90 ^{0}C. Finally, the tubes were cooled and the absorbency was taken at 660 nm by a Hitachi model 200-20 UV-VIS spectrophotometer, Germany, against a blank. The carbohydrate content was calculated by comparing the absorbency with a standard curve of d-glucose.

Study of biotic and abiotic factors

To isolate and identify the microorganisms causing spoilage in Mahua flowers, two characteristic growth media were utilized. Samples of NPS and LPS (1 g) were taken out simultaneously to determine growth of microorganisms, if any. NPS flowers stored in normal environmental condition of storage were kept out and cut into small pieces under aseptic conditions. Then, cut pieces of NPS samples were separately sprinkled on the characteristic growth media described below to isolate the bacterial and fungal components. Simultaneously, powdered mass of LPS were also put aseptically on the characteristic growth media and evenly shared out using a spreader. This process started just after harvesting season and continued up to the next year's harvesting time.

Preparation of media

Medium for Bacteria culture:

31 g of Nutrient Agar or NA (HiMedia Laboratories) dissolved in distilled water and the final volume was made up to 1000 ml. The pH of the above medium adjusted to 7.4. The flask containing medium was plugged and autoclaved at 15 lb/cm^2 steam pressure for 15 minutes.

Medium for Fungal culture:

41 g of Potato Dextrose Agar or PDA (HiMedia Laboratories) dissolved in distilled water and the final volume was made up to 1000 ml. The pH of the above medium was adjusted to 5.6. The flask containing, medium was plugged and autoclaved at 15 lb/cm^2 steam pressure for 15 minutes. These characteristic media were used for isolation of fungi and bacteria that might have occurred with the samples.

Preparation of Petri plates and culture method:

A number of pre-cleaned, dried, sterilized Petri plates kept in the hot air oven at 50^0C were taken out for culture of the microorganisms. The culture media prepared earlier were poured aseptically in a culture room under the laminar flow. The characteristic growth media for fungi and bacteria were poured into each Petri plate and properly marked. The media containing Petri plates were allowed to solidify and the cut pieces of NPS and powdered mass of LPS were separately sprinkled to separate Petri plates with the help of a sterilized glass spreader. After the inoculation of the samples, the Petri plates were transferred to incubator at 25^0C ($\pm1^0$C) for 72 hours in an inverted position. At every one-day interval the growth of the colonies, if any, were marked. Numbers of bacterial and fungal colonies were counted in five replicates of Petri plates. The colonies of the organisms were isolated and then, their pure cultures were made. These were subsequently identified and preserved at 4^0C in the refrigerator for future study.

Postharvest Storage techniques

Physical methods

a) Solar radiation

Solar drying was done only under dry weather conditions when Sunlight reaches the earth uninterruptedly, i.e. when sky was not cloudy. Amounts of 50 g of NPS were taken out from its storage condition spread uniformly in trays under the direct sunlight. Care was taken not to overcrowd the flowers so that each flower uniformly received the sunlight. The exposure to sun for drying was allowed for five hours from 11 AM to 4 PM. This process was repeated in every fortnight intervals. These samples were allowed to grow on NA and PDA media as described earlier.

b) UV-irradiation

NPS were taken out from the storage condition, cut into small pieces, 50 g amounts taken and spread properly in a tray in the UV Chamber (60 watt tube lamp) for two hours. NPS were spread under the light source at a distance of approximately 60 cm. The UV-light was switched on half an hour before the operation in order to sterilize the room perfectly.

Chemical treatments

a) Antibiotics

The culture media prepared as described earlier were mixed with streptomycin and penicillin separately in a concentration of 50 mg l^{-1} each. The sterilized media were transferred to sterilized Petri plates aseptically under laminar flow and allowed to solidify in the culture room. Cut pieces of NPS were sprinkled aseptically into each Petri plate with antibiotics included separately. Again, the microorganisms isolated and preserved in pure form earlier from NPS were transferred into the Petri plates with antibiotics, separately. Then, Petri plates were sealed and incubated in an incubator at 25^0C ($\pm1^0$C). After the desired period of incubation Petri plates were checked to determine growth of any microorganisms.

b) Liquid nitrogen

Liquid nitrogen is normally stored in a specially constructed container. Here, the freshly collected Mahua flowers were washed and then dried completely in a hot air oven at 50^0C. These dried Mahua flowers were grinded with liquid nitrogen using mortar and pestle. The whole process was conducted inside the culture room where the temperature was approximately 10^0C ($\pm1^0$C). The powdered samples were packaged in different airtight sterilized vials and stored in the deep freezer at 0^0 C ($\pm1^0$C).

c) Nitric acid

The Mahua flowers were taken in 60 g amounts and allowed to oxidize with nitric acid (HNO_3) in the presence of catalysts (V_20_5 /Fe_3+) in a beaker. The reaction was carried out at the temperature of approximately 60^0C with proper agitation and heat transfer arrangements.

$$C_6H_{12}O_6 + 12HNO_3 \xrightarrow{V_20_5\ /Fe_3+} 3C_2H_20_4 + 5H_2O + 3NO + 9N0_2 + 292\ \text{Kcal/mole.}$$

Results

The carbohydrate content was 54% approximately (Fig. 7) in LPS without any deterioration, whereas significant deterioration in NPS occurred throughout the year of storage. Approximately 70% deterioration in carbohydrate content was marked in NPS following microbial growth. Further it was observed that high humidity with slight lowering in temperature increased deterioration rate from June (90 days) onwards (Fig. 8) during storage period. NPS showed spoilage mainly due to abiotic and biotic factors following deterioration in carbohydrate content (Fig. 8). The abiotic factors responsible were temperature and moisture but LPS didn't show any spoilage during storage (Fig.1).

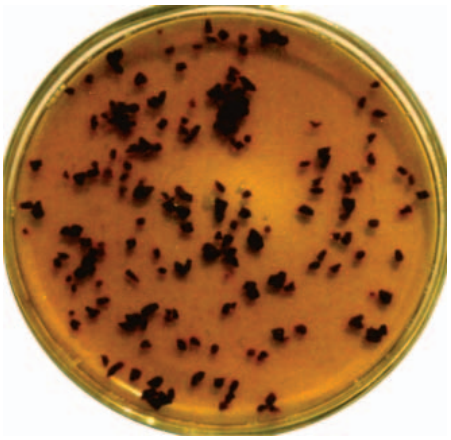

Figure 1. No microbial growth in LPS

Figure 2.
Microbial growth in NPS

Figure 3.
Microbial growth in NPS

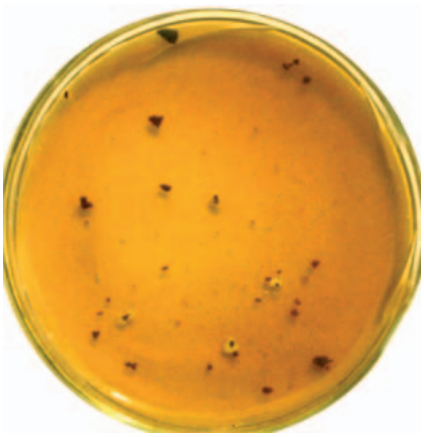

Figure 4.
Microbial growth in UV & sundried flowers

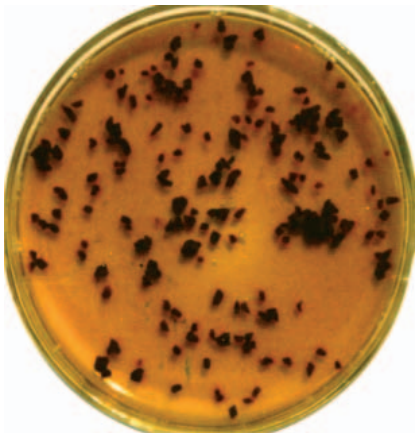

Figure 5.
No microbial growth in antibiotics treated flowers

The biotic factors causing spoilage were isolated and identified as *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus stolonifer* and *Penicillium citrinum*. Besides, two Gram positive bacteria were causing deterioration in NPS (Fig. 2, 3, 4, 5) (Table 1 & 2). More fungal colonies were observed than bacterial colonies throughout the year of storage (Fig. 6). It is also marked that during rainy season the deterioration rate was significant with higher number of microbial colonies found responsible for lowering carbohydrate content (Fig. 7 and 8).

To check spoilage or deterioration, various storage techniques i.e. physical and chemical treatments were experimented. It was determined that solar radiation could not inhibit microbial growth (Fig. 4). The UV-irradiated flowers in the characteristic culture media showed no trace of microorganisms but inciting biotic agents reappeared when the treatment was not carried out at frequent intervals (Fig. 4). However, treatment of antibiotics and liquid nitrogen showed no trace of microorganisms in NPS (Fig. 5). Trace of the microbes could not be detected at any stage of the investigation using LPS in the culture media (Fig. 1). It was further detected that inoculation of earlier preserved pure cultures of microbes into the culture media with antibiotics exhibited no microbial growth. Again, conversion to oxalic acid form was good in production and purity aspects (Tab. 3).

Discussion

A conscious attempt has been made from the beginning of the investigation to maintain the original quality of the flowers. One set of samples was stored following traditional method (Jerkin, 1982) and used it (NPS) as per the necessity and other set was processed by an indigenously developed method of the labora-

Table 1. Bacteria isolated from NPS flowers

Type of bacteria	Color	Form/margin	Elevation/shape	Growth	Remarks
Gram positive	Whitish yellow	Shining, oval	Flat, continuous	Good, patches	Coccus
Gram positive	Brown	Oily, irregular	Flat, continuous	Good, patches	Coccus

Table 2. Fungi isolated from NPS flowers

Type of fungus	Color	Texture	Mycelium	Growth	Type of reproductive organ
Aspergillus flavus	white	velvet	septate	well delicate	conidiophores
Aspergillus niger	black	smooth	septate	well delicate	conidiophores
Penicillium citrinum	greenish-blue	leathery	septate	well developed	long erect conidiophores
Rhizopus stolonifer	brownish	velvet	coenocytic	well developed	long erect sporangiophores

Table 3. Oxalic acid production from Mahua flowers

Amount of Mahua flower	Ratio of acid mixture to Mahua flowers	Oxalic acid yield in gm (w/v)	Purity of Oxalic acid (%)
60 gms	8.0	32	99.5
60 gms	10.0	36	99.5
60 gms	6.0	30	98.0
60 gms	12.5	38	99.0
60 gms	7.5	32	99.5

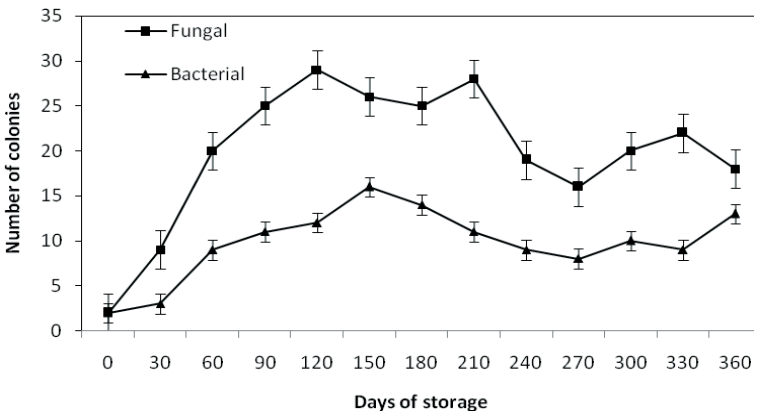

Figure 6. Number of bacterial and fungal colonies counted on culture Petri plates of NPS. Data are the mean of five replicates with ±S.E.

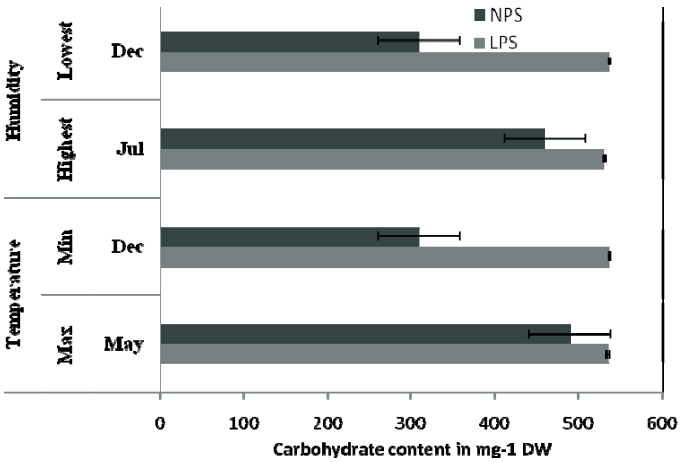

Figure 8. Carbohydrate content at highest and lowest humidity and temperature during storage of LPS and NPS. Data are the mean of three replicates with ±S.E.

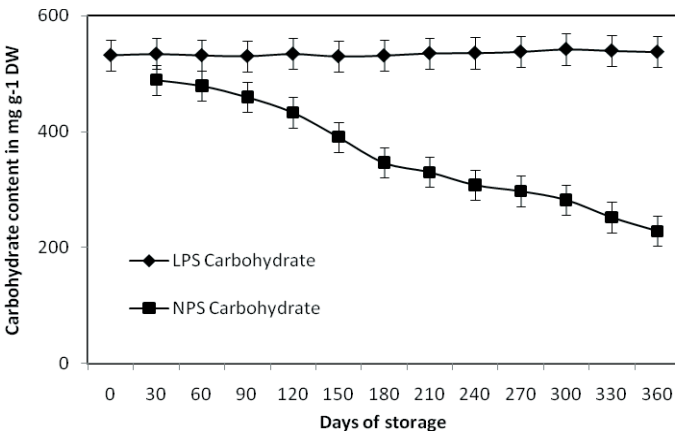

Figure 7. Total carbohydrate content during storage of LPS and NPS. Data are the mean of three replicates with ±S.E.

tory (LPS). Two types of parallel samples were made in order to carry out the work uninterruptedly throughout the year of storage (Das et al., 2010). Accordingly, stored NPS were taken out intermittently and exposed to the bright sun light on concrete floors, as a traditional storage practice followed by its collectors. The flowers actually get dried by this method and thereby, easy spoilage is averted (Olandiram and Iwv, 1993; Varghese et al., 2002; Lee and Resurreccion, 2006). In order to handle such situation, LPS were stored at low temperature following Mohammed et al. (1996) and Concellon et al. (2007). Powdering with liquid nitrogen and low temperature storage hindered growth of microorganisms. Hence, there was no spoilage during storage (Das et al., 2010) (Fig. 1). However, NPS showed spoilage (Fig. 2 & 3). Just after harvesting season, this part of the world experiences rainy season when environmental temperature get lowered to 30-32^{0}C with increased moisture content. This is the most favourable condition for microbial growth and proliferation (Fig. 6). In the most cases the microorganisms use the food as its nutrients source for growth and proliferation. By increasing their numbers utilizing nutrients, producing enzymatic changes and contributing off odor by breakdown of products or synthesis of new compounds, the invisible microbes cause spoilage, decay and deterioration of the nutrients (Mowlah and Itoo, 1983; Rodriguez et al., 1999). Again, the presence of rich carbohydrate content might have added to the process of spoilage and damage of the flowers more easily as studied earlier (Sanderson and Spotts, 1995; Sholberg et al., 1996; Skinner and Banfield, 1997; Pace, 1999). Added, with the onset of rainy season the rate of carbohydrate content deterioration was higher as environment became more suitable for microbial growth and proliferation.

The microbes isolated and studied according to their color, mycelium, texture, growth, reproductive organ etc. and the fungi (Fig. 2; Fig. 6) grew luxuriantly by utilizing the nutrients, producing various enzymatic changes and contributing of flavour by breaking down forming products (Chaitanya et al., 2000a; Errampalli et al., 2006). Specially, microbes used ascorbic acid, a potential and effective scavenger of free radicals for its own defence system under high moisture content. Besides, two Gram positive bacteria, which resemble yeasts have been identified here, might be causing fermentation of the flowers (Table 1).

Since the flowering occurs only once in a year, the development of proper storage techniques felt necessary. Physical treatment i.e. sun drying is an old and traditional practice of storage. In the present investigation, the practice could not inhibit the growth (Fig. 4) of microbes as the drying could not reduce the available moisture content to a great extent, in NPS. Another physical treatment i.e. UV-irradiation, is most widely used in food industries (Doyle, 2000; Mariam et Al., 2009). Radiation near wavelength 260 nm is absorbed strongly by purines and pyrimidines, thereby it is mostly germicidal (Luther, 1999). In present investigation UV-ray treatment completely inactivated the microbes when treated but later on, microbes reappeared on treated flowers (NPS). This has happened because of ubiquity of microbes and reactivation of established microbes with certain modifications in their characters (Fig. 4). These attributes of the microbes during storage have been marked by a number of workers (Aidoo, 1993; Zagory, 1999; Lopez et al., 2007; Mariam et Al., 2009).

The use of antibiotics is a well-established practice to protect stored grains against microbial spoilage and damage (Eckert and Ogawa, 1988; Ligia et Al., 2008). It showed positive results by inhibiting bacterial and fungal cell wall development, their enzymatic activity and action mechanism (Debano and Gordee, 1994; Arthur, 1996; Errampalli et al., 2006). However, the optimization dosages of antibiotics are cumbersome and time-consuming affairs (Levy, 1998). Again, the economic aspect and adverse impact of antibiotics usage on human beings are to be properly assessed before their application.

In the 2nd chemical treatment i.e. liquid nitrogen treatment was the most effective and a completely new step towards food storage in less storage space. It completely dried the flowers and their preservation inside the deep freezer did not allow microbial growth (Table 1) due to very low temperature and moisture free environment (Yaman and Bayindirh, 2002; Ismael and Pedro, 2009). Although a number of workers have stored in modified environment (Mohammed et al., 1996; Kappel et al., 2002; Ligia et. al, 2008) but this type of specialized storage condition-modified atmospheric packaging (MAP) with treatment is new one and did not show spoilage (Das et al., 2010).

The 3rd type was Nitric acid (HNO_3) treatment that resulted in oxalic acid formation (Table 3). It is used as a dye and drug intermediates in the application of dye stuffs to textiles, metal cleaning and treatment, permanent press, wrinkle resistance fabrics, plastics, photography, food industries etc (Hosttettmann et al., 1998). In India, major raw material for Oxalic acid is sugarcane, in view of the occurrence of more sugar in Mahua flowers it constitutes an alternate potential to oxalic acid production. Again, oxalic acid is largely used as a preservative and no loss was marked during its storage. It also requires less space for storage and when necessary it can be sold without any loss in market value.

Conclusion

Liquid nitrogen treatment was the most effective and a new step for storage of Mahua flowers. Next to it is the oxalic acid form of the flowers that has beneficial effects for varied uses. These findings are unique proper storage of such carbohydrate rich flowers on large scale and it would definitely strengthen the financial status of its collectors. Attention should also be diverted for manufacturing of more alcoholic beverages from Mahua flowers due to presence of high amount of sugar content. The retention of the quality of the flowers during postharvest storage thus should be the principal concern. The work done here is a step in achieving that objective. Upon further assiduous analysis, the information obtained and technologies developed will no doubt act as the catalyst to solve hunger and malnutrition to a great extent.

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