CHAPTER 2

REVIEW OF LITERATURE

1. Botanical characteristics of longan

Longan or dragon eye fruit (*Dimocarpus longan* Lour.) is a subtropical fruit in Sapindaceae family like litchi and rambutan. The origin is uncertain; some said the mountainous chain in Myanmar through Southern China while others said southwest India and the lowlands of Sri Lanka including (Wong and Ketsa, 1991). The crop is mainly grown in southern China, Taiwan and Thailand. Smaller growing areas are found in Vietnam, Cambodia, Laos, Queensland (Australia) Indonesia and Florida (The United States of America). Since the ancient time, the Chinese have grown longan in the south, especially in the provinces of Fujian, Guangdong and Guangxi (mentioned in literature in the period of Emperor Cheng Tang in AD 1223). Longan growing extended to India, Sri Lanka, and Myanmar then to Australia, the United States of America (Florida and Hawaii), Cuba, West Indies, and Madagascar in later years. (FAO, 2007)

In 1896, the Chinese merchant brought longan's trees to Thailand and it is distributed in the northern part and the other parts of Thailand now. Longan had been classified into 2 types; according to the growth habit, characteristics of the fruit, aril, seed and taste, as climbing longan (*Dimocarpus longan* var. obtusus Leenh.) and tree longan (*Dimocarpus longan* Lour.) (Subhadrabandhu, 1990)

The botanical characteristics of longan had been reviewed by Groff (1921), Leenhouts (1971), Subhadrabandhu and Yapwattnaphun (2000a) and Menzel *et al.* (2000). Longan is an evergreen tree that can grow up to 12 – 15 m in height, and has a spreading or erect habit, depending on the cultivar. The trunk is brittle, with branches having corky bark that splits and peels. The compound leaves are alternate and pinnate with 3 – 5 pairs of leaflets. They are 3 – 6 cm in width, 7 – 15 cm in length and glossy dark green in color on the adaxial but paler green on the abaxial.

The young leaves are red-brown, becoming light to dark green when mature. The inflorescences are compound dichasia with many branches borne on terminal shoots, 15 – 60 cm in length.

Flowers are white in color. There are three types of flowers. The male flowers (Type I) have about 6 – 8 hairy stamens arranged in a single row on a light-brown disc. Each stamen has a two-lobed anther. The anther is longitudinal dehiscent. The female flowers (Type II) contain bicarpellate hairy ovaries with a bilobed stigma. Normally only one locule in each female flower develops. The anthers have short filaments and are sterile. The perfect flowers or hermaphrodite flowers (Type III) have eight stamens with sessile filaments and produce viable pollen.

The fruit is a simple fruit. A berry is spherical to ovoid with the pericarp which develops from ovary wall. The peels are reddish brown or light brown, thin, smooth or nearly smooth. The aril is composed of parenchymatous tissue and develops from funiculus. It is whitish, translucent or pale pink. The seeded fruit is medium thick to thick. There is only one seed in a fruit. Seed shape is globular and shiny, brown to dark brown in color.

2. Flower induction in longan

2.1 Alternate bearing in longan

Longan is an alternate bearing fruit tree like other fruit tree species such as lychee and apple. The alternate bearing phenomenon has encountered to be a problem of woody fruit trees (William and Edgerton, 1974). A heavy fruit load in one year is reflected in a strong reduction of flower production and fruit yield for the following season resulting in "on and off" years with respect to fruit load (Tromp, 2000). The reduction of carbohydrate and nitrogenous reserves in roots of the trees with a heavy crop load has also been put forward as a cause of reduced flowering and, therefore, directly linked to alternate bearing (Goldschmidt and Golomb, 1982). Reducing vegetative growth and strong sinks, such as fruit thinning, in the overbearing season can enhance flowering in the following season (McArtney *et al.*, 1995).

2.2 The hypothesis of flower induction

The hypothesis of flowering in longan is a mystery, although it was already found that flowering of longan could be induced. There are 3 concepts that can explain flowering phenomenon.

2.2.1 Florigen concept: The florigen concept is based on transmissibility of substances or signals across grafts between a "donor" shoot and vegetative recipients. It was proposed of that the florigen, a flower-promoting substance, gets produced in mature leaves under favorable conditions and is transported via phloem to a competent meristem (Evans, 1971). On the other hand, flowering promoting signals originating in roots are presumably transmitted in the xylem with the transpiration stream to a shoot meristem (Bernier *et al.*, 1993). The florigen was produced in leaves under suitable photoperiods and temperature condition before translocation to apex and induces flowering later (Marentes and Grusak 1998; Sakuth *et al.*, 1993).

2.2.2 Assimilate diversion hypothesis: This concept is based on nutrition diversion hypothesis. It pertains to the relationship between vegetative and reproductive development and a notion that a critical part of the shoot apical meristem is relatively deprived of nutrients during reproductive development or must receive a higher level of assimilates for gene expression than required for vegetative development. The class of chemicals, climatic conditions and managements that mobilize nutrients at shoot apical meristematic tissues or suppress competitive sinks for assimilates at times appropriate for floral initiation assume greater importance in the nutrient diversion hypothesis. Thus auxins, cytokinins and gibberellins should increase assimilate transport towards the kind of treated tissues and may promote or inhibit flowering depending upon the tissues treated and their specificity of action (Sachs, 1977).

2.2.3 Multifactorial control: This hypothesis postulates to several chemicals, assimilates and known phytohormones participating in floral induction as promoters or inhibitors (Bernier, 1988; Bernier *et al.*, 1993). Phytohormones have

been reported to promote or inhibit flowering in many fruit trees, such as gibberellins can affect in inhibiting flower bud induction (Goldschmidt *et al.*, 1997) but cytokinins have been reported to promote flowering (Skogerbo, 1992; Stern *et al.*, 2003).

However, all of these hypotheses are based on two factors i.e. external and internal factors.

2.3 External factors

External factors influence flowering in many plant species, especially, having effects on timing of flowering. Environmental factors such as temperature, light, relative humidity, plant nutrient elements, and water, affect flowering process in longan.

2.3.1 Temperature: Longan requires a period of minimum low temperature to induce flower initiation following by panicle development. However, they are sensitive to frost and are killed or severely injured by prolonged temperatures below freezing (Menzel *et al.*, 1995). Wong and Ketsa (1991) described longan as a subtropical tree that grows well in the tropics but requires a prominent change of seasons for satisfactory flowering. A short (2-3 months) but cool (mean temperature of 15-22°C) winter season induces flowering. Menzel *et al.* (1989) reported that longans grow and crop best in areas with short, cool, frost-free winters and long, hot, humid and wet summers. Yaacob and Subhadrabandhu (1995) reported that environmental factors are important to flowering and fruit setting. Long cool seasons help in flowering and fruit setting while hot dry weather is common in causing poor setting, and dropping of fruits. Nakasone and Paull (1998) have diagrammatically presented the longan fruiting cycle and climatic and environmental clues that influence flowering (Figure 1).

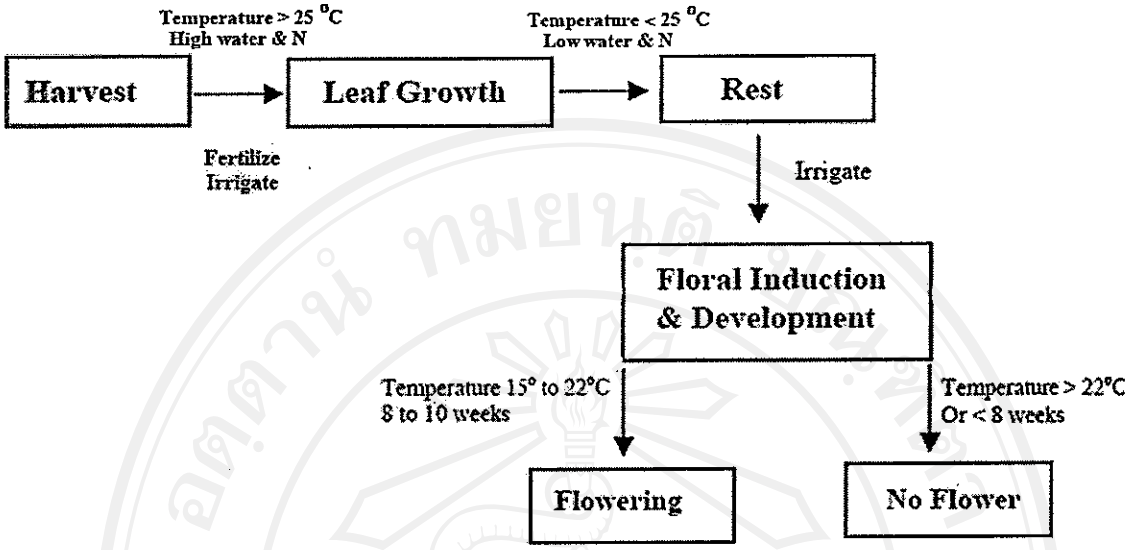

Figure 1 Fruiting cycle of longan as affected by temperature, nitrogen (N) fertilization and soil water availability (modified from Nakasone and Paull, 1998)

2.3.2 Photoperiod: This is a cycle of day length within a 24-hr period. Flowering of a plant in response to photoperiod is known as "photoperiodism". Plants can be classified as a short-day, long-day, or day-neutral plant. A short-day plant is the one in which flowering takes place when the day length is shorter than a critical value (and number of hours of day length per day), while a long-day plant is the one in which flowering takes place when the day length is longer than a critical value. A day-neutral plant is the one in which flowering is not affected by photoperiod. Even if longan is the day-neutral plant but light has direct effects on photosynthesis pathway and accumulation of carbohydrate (Menzel *et al.*, 2000).

Temperature and photoperiod interact. They individually play roles in bud initiation in some plant species, effecting induction of flowering. Critical photoperiod can also be affected by varying temperatures (Tompsett, 1976). This phenomenon can be shown in flowering in some ornamental plants such as Primula (*Priumula obconica* Hance) (Karlsson and Werner, 2002).

The other factors were relative humidity, water, nutrition and cultural practices. In longan before flowering, relative humidity decreased which caused

reduction of carbohydrate metabolism. Therefore, the accumulation of carbohydrate increased (Menzel *et al.*, 1989). Hsiao (1993) suggested that during severe water stress at the time of anthesis, pollination could be prevented and the number of reproductive sinks for assimilates were reduced. However, mild to moderate water stress before and during anthesis could enhance the partitioning of assimilates toward reproductive sinks. Increased partitioning of assimilate to the reproductive sinks promoted embryo development which in some species could result in early maturation of a portion of the fruit. Menzel *et al.* (1995) found that before anthesis in lychee trees, tree water status did not related to extension growth of floral panicles or leafy shoots. In contrast, they found that vegetative shoots were not initiated after fruit set in drought-stressed trees when water potential declined to -2.5 MPa. On predawn, the leaf water potentials of -1.7 to -3.5 MPa resulted in reduced growth but did not induce flowering in avocado and lychee compared to trees with leaf water potentials of -0.4 to -0.7 MPa (Chaikiattiyos *et al.*, 1997). Longan is tolerant of dry soil conditions. Withholding or reducing irrigation during the late summer or early fall through winter is recommended to stop or reduce excessive vegetative growth and enhance subsequent flowering during the spring (Crane *et al.*, 2005)

Ungasit *et al.* (1999) suggested that trees should be pruned, fertilized (high N) and irrigated immediately after harvested to induce new leaf growth. Soil moisture should be lowered and nitrogen fertilizer applications uphold until flowering or two months before then to allow the mature flush to "rest". Pre-flowering fertilizers should contain high P and K. After flowering and a month prior to harvest, fertilizers with high N and P and high K were recommend. It was reported in Thailand that cincturing (of branches and stem) could induce dormancy and gave rise to better flowering, fruiting and production. However, the results did not consistent and it could not be applied commercially. The easy-to-flower cultivar "Phetsakorn" could be induced to produce early and uniform flowering by cincturing of branches or stems (Subhadrabandhu and Yapwattanaphun, 2000b).

2.4 Internal factors

Internal factors, i.e. plant nutrient contents, phytohormone and genetic factors, have major effects on flower induction.

2.4.1 Plant nutrient contents: Plant nutrition is proposed to be one of the internal factors. It was found that nitrogen and carbohydrate in shoots influenced vegetative and reproductive growth (Urban *et al.*, 2004). Zee *et al.*(1999) reported that the most effective method to prevent vegetative growth in litchi was to maintain leaf N content at 1.75 – 1.85 % by applying nitrogen fertilizer only after panicle emergence and fruit set. Nitrogen sensing appeared to regulate varieties of physiological and developmental processes in plants. Nitrate activated transcription of genes that were involved in nitrate transport (Coruzzi and Bush, 2001). Expression of NR (nitrate reductase) and NiR (nitrite reductase) genes were induced by nitrate. Nitrate was known to regulate via the intermediate of the glutamine and glutamate ratio, the phosphorylation and activity of PEPase (phosphoenolpyruvate carboxylase), SPS (sucrose phosphate synthase) and NR enzyme that controled the distribution of photosynthetic carbon between the synthesis of sucrose and amino acids (Limani and Ameziane, 2000). The carbohydrates in plant were sugars, mainly glucose or sucrose. The evidence that sucrose might function in long-distance signaling during floral induction came from studies of *Sinapsis alba,* a long-day plant in mustard family. After induction of flowering by either a single long day or by a displaced short day, the concentration of sucrose in the phloem reaching the shoot apex increased rapidly within one hour of the photo extension for a long day treatment and transiently. This pulse of sucrose translocation normally just before the increase in cell division was observed in the shoot apical meristems during floral initiation (Bernier *et al.*, 1993). In the fruit tree such as litchi, Menzel *et al.* (1989) reported that the flowering and starch content in litchi were correlated. So, the cultural practices which induced stress condition such as girdling increased accumulation of carbohydrate. However, there was an evidence that high starch level promoted flower initiation (Whiley *et al.*, 1989). Yamasaki *et al.* (2002) found the involvement of carbon and nitrogen allocation in the flowering process of the strawberry cv. Toyonoka induced by low nitrogen by using ^{13}C- and ^{15}N-tracers. The allocation pattern of C and N in flower-

induced plants by low N was marked by 1) a carbon content that was also low, 2) a recently fixed C that was primarily allocated to roots and 3) a recently absorbed N that was allocated more to crowns, young leaves, and shoot apices than it was to leaves.

The interaction between C and N metabolites in higher plant cell is governed by many regulatory factors. The coordinate of C and N metabolism is reflected by the complex interplay between signals involving carbon metabolism, such as sucrose and light, and those associated with nitrogen metabolism, such as nitrate (Lancien *et al.*, 1999). C:N ratio sensing mechanism enables plants to activate genes involved in N assimilation when carbon skeletons are abundant and internal level of organic-N are low, or to hault N- assimilation when levels of photosynthate are low or internal levels of organic-N are high (Gocal *et al.*, 2001). Carbon and nitrogen metabolisms are tightly linked and it seems obvious that nitrogen signaling pathways interact with sugar signaling pathways. Such the interaction in turn may be controlled or mediated by phytohormones. Glucose signaling in plants has been shown to involve complex cross with hormone signaling pathways (Ohta *et al.*, 2001). Carbon metabolite was found as a signal to link to ethylene, abscissic acid (ABA) and GA response pathways (Coruzzi and Bush, 2001).

2.4.2 Phytohormone: Phytohormones or plant hormones are internally secreted chemicals in plants that are used for regulating the plant growth. According to a standard definition, plant hormones are signal molecules produced at specific locations, occur in low concentrations, and cause altered processes in target cells at other locations. In longan, Lin *et al.* (2001) found that the content of indole-3-acetic acid (IAA) were high during staminate differentiation and low during pistillate differentiation. The differentiation of flowers was accompanied by an increase in gibberellic acid (GA_{1+3}). Abscisic acid (ABA) was low before sexual differentiation, but increased at anthesis. The ratio of (IAA+ zeatin (ZR) + GA_{1+3}): ABA increased during female flowering, and then decreased at anthesis. A lower ratio appeared to benefit female flowering. However, the flower bud differentiation of longan did not occur until shoots elongated. The morphogenesis of apical and axillary meristems proceeded independently with leaves in some inflorescences. The concentrations of

cytokinins and abscisic acid increased but gibberellins decreased. Cytokinin and abscisic acid concentrations promoted flower bud morphogenesis while gibberellin inhibited it (Qiu *et al.*, 2004).

2.4.3 Genetic factor: The genetic factor is an internal factor that a few understood of its mechanism until the last decades. The genetic model of *Arabidopsis thaliana* is widely used. The flowering of *Arabidopsis* is a result from the perception of various environmental influences such as photoperiod, light quality, temperature and hormones (i.e. gibberellic acid, cytokinins, auxins and abscisic acid) (Putterill *et al.*, 2004). The nature in flowering of *Arabidopsis* has been attributed to differences in the sequences of key regulator genes such as FRI (FRIGIDA) and FLC (Flowering Locus C) (Salathia *et al.*, 2006)

He and Amasino (2005) found that FLC was a MADS-box transcription regulator that inhibited the floral transition by Suppressor of Over Expression Co1 (SOC1) and Flowering Locus T (FLT) which in turn activated floral-meristem identity gene such as LEAFY and APETALA1. The FLC was a primary regulator of flowering that was the convergence of the vernalization and FRI pathways. Mutations of genes that encoded components of autonomous pathway and FRI expression could inhibit flowering; vernalization reduced FRI expression, which in turn reduced FLC levels in part through the chromatin modification of FLC. The identification of the gene involved in *Arabidopsis* flowering had led to the isolate of homologous genes in other plants including MADS-box gene from wood tree species. However, the function of MADS-box genes in trees remained largely unknown and there were no direct evidence that gene regulated flowering in *Arabidopsis* (Cseke *et al.*, 2003; Brunner and Nilsson, 2004). The complete sequence of the popular genome did not contain a homolog to the *Arabidopsis*. The FLC gene suggested that tree may have alternate methods of controlling flowering. In addition to a currently unknown molecular mechanism for flowering in tree, tropical and sub-tropical fruit trees had the additional complication of not having a distinct growing season compared to their temperate counterparts (Brunner and Nilsson, 2004).

The suppressive subtractive of differentially hybridization (SSH) was a tool in identification of differentially expressed of gene involved in many complex processes. This method had successfully identified BTH responsive gene in papaya (Qui *et al.*, 2004) and reported about 65 unique longan genes identified by SSH method (Matsumoto, 2006).

3. Potassium chlorate and flowering induction of longan

3.1 Potassium chlorate property

Potassium chlorate is an inorganic salt, oxidizer group. The other name of potassium chlorate are chlorate of potash, Bertholler salt and potassium oxymurate. It composes of potassium (K), chlorine (Cl), and oxygen (O) and molecular formula is $KClO_3$. Potassium chlorate is colorless crystal and can be grinded to white powder. Potassium chlorate reveals the physical properties as molecular weight 122.55 g/mol; specific gravity 2337; melting point 356°C; boiling point ~400°C; solubility in water 7.3 g/100 ml of water at 20°C (Wikipedia, 2007a). It is moderately toxic, forms explosive mixtures with combustible materials such as sulfur, sugar, etc, and is a strong oxidizing agent. It could be hydrolyzed to potassium ion (K^+) and chlorate ion (ClO_3^-) as appears in the equation below.

$$KClO_3 \longrightarrow K^+ + ClO_3^-$$

3.2 Potassium chlorate in plant

Potassium chlorate dissociates into potassium ion and chlorate ion. Potassium chlorate diffuses into roots and then mobilizes through xylem. Since chlorate ion and nitrate ion (NO_3^-) are analouges so they both respond to nitrate reductase (NR). NR can be used to detect mutants which are deficient in nitrate reductase (Wilkinson and Crawford, 1993). Nitrate reductase readily reduces chlorate to chlorite which is toxic (Figure 2).

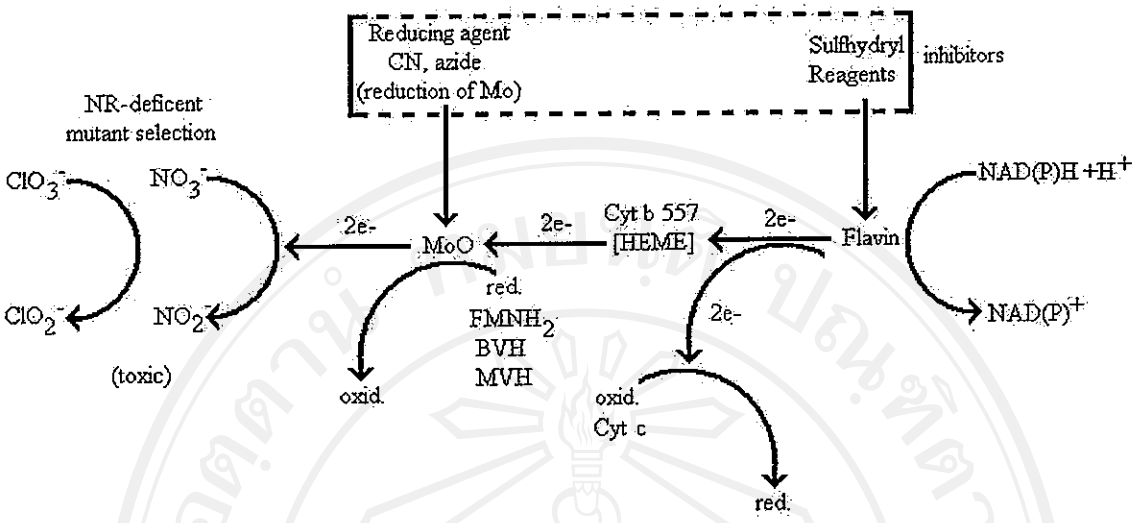

Figure 2 Mechanism of reduction of nitrate and chlorate in plant (Modified from Lin *et al.*, 2001)

Earlier, a chlorate resistant mutant (*chll*) of *Arabidopsis* had been identified which was defective in nitrate transport, but this might be the low-affinity nitrate transporter. Chlorate might not be a useful analog for the root high – affinity nitrate transport system (Kosola and Bloom, 1996). Light acted both directly as a signal and indirectly through photosynthesis to regulate the expression of genes encoding nitrate reductase (NR). A chlorate-resistant mutant of *Arabidopsis* (*cr88*) had recently been isolated which was defective in the regulation of NR gene expression. The response of *NR*, but not *NR1* or the gene encoding nitrite reductase (NiR) was impaired in *cr88*. The light regulation of the genes encoding the chlorophyll a/b binding protein and the small subunit of ribulose-bisphosphate carboxylase/oxygenase was also impaired. The defection in red-light mediated deetiolating and nitrate and sucrose induction processes were retained in *cr88* (Lin *et al.*, 2001).

3.3 Potassium chlorate in plant physiology

Sritontip *et al.*, (2005) found that the plants treated were with $KClO_3$ had efficiency of photosystem II (Fv/Fm) at the 1st week before terminal bud break, the net CO_2 assimilate rate at the 1st week before terminal bud break and the net CO_2 assimilate rate during terminal bud break and the transpiration rate were higher than

the untreated plant. Potassium chlorate had an effect on total nitrogen, it increased but total non structural carbohydrate tended to decrease before flowering (1^{st}-3^{rd} week) then increased after flowering. The contents of cytokinin-like substances were higher but gibberellin-like substance contents were lower (Wangsin and Pankasemsuk, 2005). Hegele *et al.*, (2006) found that $KClO_3$ affected photosynthesis in leaves and endogenous hormone levels in terminal buds. It reduced photosynthesis and auxins but increased cytokinin contents.

3.4 Effect of potassium chlorate on flowering in longan

In 1994, a farmer in the southern part of Thailand found that sodium chlorate could induce flowering in longan while 3-4 years later, longan growers in Lamphun province also found the same thing. The inflorescence emerged in most treated trees about 20 to 25 days after potassium chlorate application. However, some trees showed declining symptom such as yellow leaves. Some trees sprouted some shoots without flowering (Subhadrabandhu and Yapwattanaphun, 2000 b).

When chlorate ion is uptaken by xylem in longan trees, it competes with nitrate ion for nitrate reductase and nitrite reduction so the reduction of nitrate is interrupted. Ammonium ion has diminished and amino acid and protein are decreased too. On the other hand, chlorate ion is reduced by nitrate reductase and chlorite ion is reduced by nitrite reductase. The product that occurred will cause DNA methylation and gibberellins synthesis is later reduced (La Brie *et al.*, 1991).

The effectiveness of potassium chlorate in flowering induction in longan are based on the stage of leaf development, amount of the chemical, , duration of application and soil texture (Thaping-Kae, 1999). Manochai *et al.* (1999 a) found that the optimum stage of leaf development was at 45 days old, approximately. The amount of potassium chlorate used depended on the size and age of longan trees. General recommendation for potassium chlorate application was 5-10 g per square meter of canopy by soil drench or 100 – 3,000 ppm in foliar application (Pankasemsuk, 1999). Trees grow in sandy soil responded better than those grown in heavy clay. Potassium chlorate application, as soil drench was the most effective

method to induce flowering in longan. A significant reduction in photosynthesis was observed about 10 days after application (Hegele *et al.*, 2006).

4. Molecular methodology in plant physiology

4.1 Molecular methodology

4.1.1 Isozyme and protein: The isozymes are similar forms of the same enzyme that share identical functions. There are different types of isozymes that are classified according to their origin. The isozymes may be encoded by several different loci (which determine subunit structure), or they may be determined by a single locus with several different alleles. Many well-known enzymes and their isozymes used in electrophoretic studies function in the metabolic pathway. Enzymes are a kind of protein that composed of an intertwining string of amino acids, give it a specific molecular structure. It is this structure that is crucial to the enzyme's ability to function. The structure of the enzyme is directly affected by hydrogen bonds and charge-relationships within the amino acid chains. All of them are neutral charged excepting aspartic acid and glutamic acid which have negative charges and lysine and arginine are positively charged (Weeden, 1983; Wendel, 1989).

4.1.2 Isozyme and protein electrophoresis: The basis of electrophoretic analysis of isozymes was started in 1957 after Hunter and Mohler discovered the isozymes (Stebbins, 1989). Buth (1984) introduced the concept of isozymes, which they defined as the different molecular forms in which proteins might exist with the same enzymatic specificity. This meant that different variants on the same enzyme had identical or similar functions and were present in the same individual. As such, their importance for understanding gene action in development and differentiation was exploited during the 1960s in animals and plants. Nevertheless, isozymes played a minor role in research on plant biochemistry until 1966 when genetic polymorphism for isozymes within the same population was discovered (Stebbins, 1989; Wendel, 1989). It revealed the possibility for population genetics to make precise quantitative estimates of genetic variability based upon one parameter of the molecular structure of the primary products of the genes themselves. Plant population genetics were not long in following their zoological colleagues and

the investigation of both animals and plants increased explosively. This application, however, necessitated a partitioning of the isozyme concept, since they only used the relevant allozyme subset, defined by Prakash (Buth, 1984) as the variant proteins produced by allelic forms of the various polymer produced from monomers specified by different loci. The development of genetic distance or similarity coefficients (Lukasová and Šarmanová, 1985) allowed the summarization of allozyme data for intersample comparisons and so quantified allozyme data were soon applied to comparative studies of taxa.

4.1.3 The function of isozymes :

The peroxidase enzyme that increased might be used to reduce free oxygen from peroxizome in leaf. However peroxidase enzyme was basic enzyme of plant but its pattern can be used for classification of longan specifically cv. Daw (Ramingwong *et al.*, 2003). Isomerase enzyme (EC 5) was grouping of enzyme that catalysed the interconversion of isomers (Moss, 2006). There were fundamental enzymes like peroxides.

The shikimic dehydrogenase (SKDH, EC 1.1.1.25) is thus involved in the biosynthesis of a precursor of the aromatic amino acids phenylalanine and tyrosine and in the biosynthesis of precursors of a series of important secondary compounds. Both amino acids were important on development of plant.

Malate dehydrogenase is an enzyme in the citric acid cycle that catalyzes the conversion of malate into oxaloacetate (using NAD^+) and vice versa (this is a reversible reaction). Malate dehydrogenase is also involved in gluconeogenesis, the synthesis of glucose from smaller molecules. Pyruvate in the mitochondria is acted upon by pyruvate carboxylase to form oxaloacetate, a citric acid cycle intermediate. In order to get the oxaloacetate out of the mitochondria, malate dehydrogenase reduces it to malate, and it then traverses the inner mitochondrial membrane. Once in the cytosol, the malate is oxidized back to oxaloacetate by cytosolic malate dehydrogenase. Finally, phosphoenol-pyruvate carboxy kinase (PEPCK) converts oxaloacetate to phosphoenol pyruvate (Wikipedia, 2007 c).

Superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. In plant, superoxide dismutase was protected from oxidative stress caused by exposure to high light intensity and low temperature (Gupta *et al.*, 1993).

Glucose-6-phosphate dehydrogenase catalyzes the first step in the pentose phosphate pathway and is a strategic point for controlling the flux through this sequence of reactions. In plants, where G6PD occurs in the cytoplasmic and plastid compartments, the enzyme has been studied most extensively from leaves and regulation of its activity has been considered mainly in relation to photosynthetic metabolism (Fickenscher and Scheibe, 1986).

4.1.4 Polymorphism and basic principles of isozymes:

Polymorphism may be defined as simultaneous occurrence within or between populations of multiple phenotypic forms of a trait attributable to the alleles of a single gene or the homologs of a single chromosome (Acquaah, 1992). In natural populations recurrent mutations of genes produce variability. There are polymorphic loci, variable in the sense described above, and monomorphic or nonvariant. The main points of the concept of isozymes perceived by researchers are summarized below:

1) Multiple molecular forms of enzyme (isozymes) are common in organisms.

2) Isozymes share a common catalytic activity. Each isozyme has a specific role in the metabolic pathway and functions in harmony with other enzymes within the organizational framework of cells.

3) Isozymes often exhibit tissue or cell specificity.

4) Molecular heterogeneity of enzyme confers flexibility, versatility and precision upon an organism in terms of metabolic functions

5) Molecular multiplicity is desirable for biological efficiency. Isozymes arise in nature by two general mechanisms, i.e. genetic and epigenetic. The source of gene multiplicity is duplication through mutation, polyploidization and chromosomal aberrations (Hoelzel and Dover 1991). Those events constitute the

present epigenetic origin of isozymes. Epigenetically formed enzymes are not considered isozymes by some researchers. Epigenetic mechanisms may be divided into post-translational addition, post-translational deletion and post-translational conformation.

On the other hand, there are four genetic mechanisms (Acquaah, 1992):

a) Multilocus system I: The different genes code for independent proteins with the same enzymatic activity. The various genes are nuclear in origin but their protein products are located in different parts of the cell.

b) Multilocus system II: It is similar to system I except that the enzymes involved are polymeric and the subunits are encoded by more than one locus.

c) Multilocus polymeric system: The enzymes display a series of polymers that consist of identical subunits.

d) Allozyme system: The term allozyme describe isozymes encoded by allelic genes. Alleles at various loci may be modified to produce isozymes that are distributed in a population according to Mendelian laws of inheritance (Weeden, 1983).

4.1.5 Principles of protein electrophoresis: Electrophoresis is a versatile biochemical technique to detect genetic variation. Protein molecules migrate in an electric field because they are charged (Hamrick and Rickwood, 1990). When an electrical gradient is applied, the molecules migrate toward the electrode with the charge opposite to their own, with the result that the initial single boundary formed by the mixture of molecules is broken into several boundaries according to the relative mobility of the mixture. This technique is useful for separating and analyzing complex proteins mixtures. Today, active media are used. The investigator can alter the porosity of medium for more effective separation of molecules that have identical charge densities but different in size. Examples of media are agarose, starch and acrylamide gels. Some isozymes resolve better when certain combination of buffers (gel and electrode) are used (Weeden, 1983). In polyacrylamide gel electrophoresis (PAGE), multiphase buffer systems employ two kinds of gel in one run: lower gel

(analyzing or separating gel) and upper gel (stacking gel) (Hamrick, 1983; Wendel, 1989). The pH of electrophoretic buffers may be manipulated within range to optimize the resolution of bands of proteins being electrophoresis. Electrophoresis operates on two fundamental and interrelated electrical principles: electrical current, which is proportional to voltage, and power, which is directly proportional to the voltage and current. The heat generated during the electrophoretic process must be dissipated because excessive heat decreases enzyme activity (Andrews 1993). The fact mentioned above combined with the duration of electrophoresis, protein concentration of samples, quality of samples, sample size, principles of staining gels (Vallejos, 1983) and protocols strongly influenced results (Acquaah, 1992; Hamrick and Godt, 1990; Hoelzel and Dover, 1991; May, 1994; Stebbins, 1989). A good analysis expects interpretation of gel patterns. The resulting banding pattern is an electrophoretic phenotype (Wendel, 1989), which usually consist of one or more colored bands for each individual analyzed. In some cases, it may be simple and consist of a single invariant band in the whole sample. In contrast, some enzymes may display complex phenotypes with 15 of more bands per individual. So a correct interpretation of banding patterns in genetic terms requires the proper determination of the pertinent factors that influence the electrophoresis' phenotype. Moreover, e.g. null alleles, intergenic heteromultimers, multiple-banded products and artifacts may act (Šnábel, 1995; Weeden, 1983). An explanation of gel patterns in genetic terms means assessing at least the mean number of alleles per polymorphic locus and effective number of alleles per locus (Hart and Daniel, 1988; Mahy *et al.*, 1997; Ollejonsson *et al.*, 1996) from this description precedes other parameters as heterozygosis (H), genetic diversity (G) or others in accordance with the design (Godt and Hamrick, 1996; Hamrick, 1983; Hamrick, 1989). There are other possible explanations only by bands variability – mixed phenotypes (Etoh and Ogura, 1981; Karkouri *et al.*, 1996; Lehmann, 1997).

4.2 Application allozymes as a tool in research

Genetic markers generally have contributed to the study of plant biology by providing methods for detecting genetic differences among individuals. There are some important ecological topics which often use allozymes as powerful

markers: Genetic relatedness within and among populations, often with relations to geographic structure. Patterns across a broad range of taxa are mostly consistent with the understanding of the effects of the breeding system (species with selfing tend to poses lower levels of genetic variation within populations), life history (longer-lived perennials tend to be more variable) and the distribution of genetic diversity within and among populations (Hamrick and Godt, 1990; Kudoh and Whigham, 1997; Mahy *et al.*, 1997; Tarayre *et al.*, 1997). There is a narrow linkage between geography and the spatial patterns of genetic variation (Newton *et al.*, 1999) as well as the genetics of plant migration and colonization (Barrett and Shore, 1989; Sun, 1997).

Mating system estimation: An alternative approach of the study to plant mating uses classifications of mating events to characterize levels of inbreeding and patterns of gene dispersal in a population and other consequences (Holm *et al.*, 1997; Noyes and Soltis, 1996; Soltis and Soltis, 1987; Soltis and Soltis, 1989; Sun, 1996; Wang, 1996; Wang *et al.*, 2005).

Genetic diversity in cloned plant species: Asexual reproduction is relatively common in plant species and can occur through a number of modes, e.g. vegetative spread, production of vegetative propagates, apomixes (Cruzan, 1998). One difficulty with the study of genotypic diversity in cloned species is the inadequacy of allozyme markers to reliably identify all genotypes present (Lehmann, 1997; Ollejonsson *et al.*, 1996; Zeidler, 1999). It is often necessary to test paternity (Cruzan, 1998) or chromosomal locations and mapping (Satovic *et al.*, 1996; Shigyo *et al.*, 1995). Conservation biology is a rapidly rising field because of effective molecular tools (Newton *et al.*, 1999). Other studies: Seed bank populations (McCue and Holtsford, 1998), environmental changes and habitat heterogeneity (Lehmann, 1997), combination with cytological studies and other method (Anderson *et al.*, 1995; Noyes *et al.*, 1995), plant pathology (Leuchtman and Clay, 1996), germaplasm collection (Lamboy *et al.*, 1996), mycorrhizal genetic variation (Karkouri *et al.*, 1996) and phytopathology (Forbes *et al.*, 1997; Ylimattiloa *et al.*, 1997).

4.3 Limitations of protein electrophoresis

Current electrophoresis detects only the amino acid sequences that results in the net charge of proteins. As sensitive as electrophoresis may be, only about a third of all amino acid substitutions can be detected by the technique (Acquaah, 1992; May, 1994). Then the amount of polymorphisms will probably be underestimated as a result of this cryptic variation. Electrophoresis is also limited by the number of available staining protocols. The protocols available are for water soluble proteins. Another possible restriction of electrophoretic analysis of genetic variation is that only the variability in the coding portions of the DNA can be sampled. Another complication is that some organisms are polyploidy in origin and some specific genes have been duplicated in otherwise diploid organisms (May, 1994). Obviously, with more loci there are more gene copies coding for a protein (Snabel, 1995). Unfortunately, there are some monomorphic species for most allozymes. On average across taxa, less than half of all loci are polymorphic. Narrow genetic endemic species and others that have experienced genetic bottlenecks often lack polymorphic loci (Parker *et al.*, 1998). Many statistical analyses in population genetics assume that phenotypic differences among allozymes are minimal and selectively neutral, but exceptions are known. Also, codominant inheritance, although generally true for allozymes, is not always observed (Wendel, 1989).