Original article

Physiological changes in relation to growth and ripening of khirni [*Manilkara hexandra* (Roxb.) Dubard] fruit

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Physiological changes in relation to growth and ripening of khirni [*Manilkara hexandra* (Roxb.) Dubard] fruit.

Abstract — Introduction. Fruit ripening is the process resulting in changes in color, taste and texture, which make the fruit acceptable for consumption. Since a wide spectrum of physiological, biochemical and organoleptic changes are involved in the development of a soft, edible, ripe fruit, we studied theses changes in an underutilized fruit, khirni [*Manilkara hexandra* (Roxb.) Dubard]. **Materials and methods**. The changes in biochemical composition, which includes chlorophylls, carotenoids, anthocyanins, sugars, starch, free amino acids, phenols and proteins, and the specific activity of enzymes such as amylase, invertase, catalase, peroxidase, pectinmethylesterase, polygalacturanase and cellulase were analyzed in the fruit of *Manilkara hexandra* at five sequential developmental stages (young, premature, mature, preripened and ripened fruit stages). **Results and discussion**. The pulp of khirni fruit tastes sour during its growth period, but turns sweet when it ripens. A decreasing trend in chlorophylls occurs simultaneously with an increase in the quantity of total carotenoids and anthocyanins. Further, an increase in the quantity of sugars, proteins and phenols occurs towards the ripened stage, but starch and total free amino acids show a decrease in their quantities. Also, khirni fruit exhibits climacteric behavior with its increased rate of respiration and ethylene production. The moderate to significant changes in the activity of enzymes such as amylase, invertase, catalase and peroxidase involved in a number of catabolic and anabolic reactions indicate that these enzymes also have an active role in the process of khirni fruit growth and ripening.

India / *Manilkara hexandra* / fruit / maturation / plant physiology / growth

Changements physiologiques au cours de la croissance et de la maturation du fruit de *Manilkara hexandra.*

Résumé — Introduction. La maturation est un processus qui aboutit à des changements de couleur, de goût et de texture, qui rendent le fruit consommable. Puisqu'une large gamme de changements physiologiques, biochimiques et organoleptiques intervient dans le développement d'un fruit mûr comestible, nous avons étudié ces changements dans un fruit sous-utilisé, le khirni [*M. hexandra* (Roxb.) Dubard]. **Matériel et méthodes**. Les changements de certains composés biochimiques (chlorophylles, caroténoïdes, anthocyanines, sucres, amidon, acides aminés libres, phénols, protéines) et de l'activité spécifique de certains enzymes (amylase, invertase, catalase, peroxydase, pectineméthylestérase, polygalacturanase et cellulase) ont été analysés dans le fruit de *M. hexandra* à cinq stades de son développement (jeune, prématuré, mature, prémûr et mûr). **Résultats et discussion**. La pulpe du khirni a un goût aigre pendant sa période de croissance, mais elle devient sucrée lorsque le fruit mûrit. Les chlorophylles ont tendances à diminuer en même temps que la quantité totale de caroténoïdes et d'anthocyanines augmente. De plus, une augmentation de la quantité de sucres, de protéines et de phénols se produit à l'approche du stade de maturité, mais les quantités d'amidon et d'acides aminés libres totaux tendent à diminuer. Par ailleurs, le khirni montre un comportement climatérique du fait d'un taux croissant de sa respiration et de sa production d'éthylène pendant sa maturation. Des changements modérés à significatifs de l'activité d'enzymes telles que l'amylase, l'invertase, la catalase et la peroxydase impliquées dans un certain nombre de réactions cataboliques et anaboliques indiquent que ces enzymes auraient également un rôle actif au cours de la croissance et de la maturation du fruit de *M. hexandra*.

Inde / *Manilkara hexandra* / fruit / maturation / physiologie végétale / croissance

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1. Introduction

Studies on the growth of fruits have a long and distinguished tradition, as they contain a variety of biochemical constituents, which play a decisive role in determining the composition and quality of fruits [1]. Fruit ripening is the process resulting in changes in color, taste and texture, which make the fruit acceptable for consumption. Since a wide spectrum of physiological, biochemical and organoleptic changes are involved in the development of a soft, edible, ripe fruit, the present study was undertaken with a view to studying these changes in an underutilized fruit, khirni [*Manilkara hexandra* (Roxb.) Dubard].

Manilkara hexandra is a small to medium sized evergreen tree growing under semi-arid conditions, in gullied and ravinous lands. It is grown as an avenue tree and also cultivated in gardens for its very sweet edible fruits [2, 3]. Its obovoid-oblong to ellipsoid shaped fruits, measuring about 1–1.5 cm wide (*figure 1*), possessing one or two seeds, are edible fresh or dried, being a good source of minerals and vitamins with low fat content [4]. The young pods are also eaten when boiled. The fruit serves as a tonic for the heart, a good appetizer, brings back consciousness, cures vomiting and has many other medicinal uses [5]. The seeds of khirni are known to contain about 25% edible oil having high medicinal importance. Also, important chemicals such as hexandrone, quercetin, quercitrin and hexandrin have been isolated and characterized in khirni [6].

A survey of the literature reveals that, in spite of its economic and medicinal value, studies dealing with the fruit of *M. hexandra* are very meager, and this is evident from the fact that the work of only a few authors could be found [4, 7]. However, the physiology of the growth and ripening of khirni fruit has not been given its due consideration. Hence the present study.

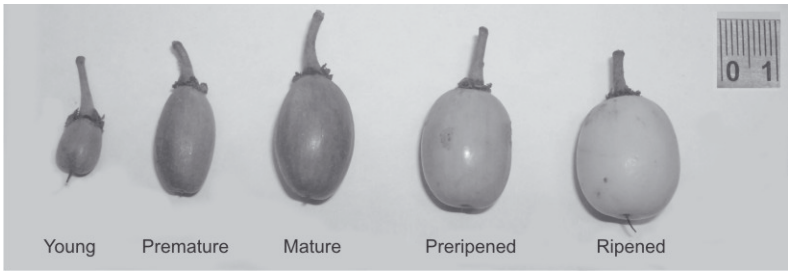

Figure 1.
Khirni fruit at its sequential stages of growth and ripening.

2. Materials and methods

Fruits of *Manilkara hexandra* were collected from the University Botanical Garden at their five successive developmental stages, *viz.* young, premature, mature, preripened and ripened. After recording the measurements of fresh weight, length, diameter, pH and total acidity of these collected fruits (*table I*), they were subjected to biochemical analyses.

The quantitative analysis of pigments such as chlorophyll *a*, chlorophyll *b*, total chlorophylls, total anthocyanins, starch, total soluble sugars, and reducing and non-reducing sugars was carried out as per the methods cited by Thimmaiah [8], while the

Table I.
Fresh weight, length, diameter, pH and total acidity of the fruit of *Manilkara hexandra* at its sequential stages of growth and ripening (values of mean ± standard deviation of five samples).

Stages of growth and ripening	Fresh weight (g)	Length (cm)	Diameter (cm)	pH	Total acidity (%)
Young	0.49 ± 0.06	0.80 ± 0.06	0.40 ± 0.06	6.8 ± 0.04	4.93 ± 0.12
Premature	0.73 ± 0.08	1.02 ± 0.05	0.77 ± 0.08	6.8 ± 0.05	4.54 ± 0.15
Mature	2.35 ± 0.11	1.67 ± 0.05	1.12 ± 0.14	6.9 ± 0.06	3.72 ± 0.15
Preripened	4.12 ± 0.14	2.10 ± 0.09	1.40 ± 0.12	7.1 ± 0.04	3.18 ± 0.14
Ripened	4.25 ± 0.16	2.65 ± 0.12	1.70 ± 0.25	7.2 ± 0.05	2.66 ± 0.11

method of Wang *et al.* [9] was followed for estimating the amount of total carotenoids.

The amount of total free amino acids was measured by using the method described by Moore and Stein [10], while the protein and total phenolic contents were determined as per the methods of Lowry *et al.* [11] and Bray and Thorpe [12], respectively.

Following the method of Teitel *et al.* [13], using the Gas Chromatograph (Perkin Elmer Autosystem XL), the rate of ethylene and respiration (evolution of CO_2) were measured at the Sophisticated Instrumentation Center for Applied Research and Testing (SICART), Vallabh Vidyanagar, Gujarat, India.

The activity of hydrolyzing enzymes (amylase and invertase) was measured as per the methods cited by Thimmaiah [8], while Devi [14] was followed for assessing the activity of antioxidant enzymes (catalase and peroxidase) and cell wall-degrading enzymes [pectinmethylesterase (PME), polygalacturanase (PG) and cellulase].

The data presented in this paper are the mean and standard deviation of three replicates for each of the parameters and were subjected to statistical analysis using Duncan's multiple range test [15].

3. Results and discussion

As the growth of the khirni fruit continues, the pH of its fruit pulp increases from 6.8 at the young stage to 7.2 at the ripened stage. In contrast, the total acidity of the fruit was found to decrease from 4.93% at the young stage to 2.66% at its ripened stage (*table I*). The change in pH according to Willis *et al.* [16] is mainly due to the leakage of organic acids from the vacuole. Further, the flavor of the fruit pulp changes from sour at the young stage to sweet at the ripened stage.

Fruit color is known to serve as an index for determining the ripening stage and optimal harvest time for various fruits. As the fruit of *M. hexandra* ripens, a visual change in its color from green to yellow occurs. A quantitative analysis of pigments revealed that the amount of chlorophyll *a* as well as chlorophyll *b* content was high (19.28 mg·100 g^{-1}) until the premature stage, but decreased towards the mature stages (13.64 mg·100 g^{-1}). Chlorophyll *b* was found to be a more stable pigment, with 13.30 mg·100 g^{-1}, than chlorophyll *a*, with 5.87 mg·100 g^{-1} at the ripened stage. However, total chlorophyll content decreased remarkably until the preripened stage (6.49 mg·100 g^{-1}) (*table II*). Perhaps, as Willis *et al.* [16] stated, this kind of decrease in the amount of chlorophyll may be due to the loss of chlorophyll as a part of a transition of the chloroplasts into chromoplasts containing yellow and red carotenoid pigments. Stanley is of the opinion that the loss of chlorophyll can also be mediated through several processes such as the action of enzyme chlorophyllase or enzymatic oxidation that produces low-molecular-weight products, which are colorless [17].

The quantity of total carotenoids of the khirni fruit is found to increase from 4.15 mg·100 g^{-1} at the young stage to 5.08 mg·100 g^{-1} at the mature stage but, subsequently, it decreases to 1.56 mg·100 g^{-1} at the ripened stage, while the accumulation of anthocyanin content reaches 2.42 mg·100 g^{-1} at the ripened stage (*table II*). Hence, anthocyanins were found to be mainly responsible for the color change during the ripened stage. These findings support the view of Stanley who stated that significant color changes may be mediated in fruits through the degradation of chlorophyll and the exposure of preexisting carotenoids [17]. Besides, the results of the present study also support the view of Singh and Sharma that unlike chlorophylls and carotenoids, which are sequestered in chloroplasts or chromoplasts, anthocyanins accumulate in the vacuoles and are responsible for change in pigmentation [18].

Sugars, either in the free state or as derivatives, play an essential role in imparting attractive color, flavor, appearance and texture to the fruits. The quantitative analysis of reducing sugars in the studied khirni fruit exhibited a two-fold increase in their quantity from the young stage (5.95 mg·100 g^{-1}) to the ripened stage (11.63 mg·100 g^{-1}) (*table II*), while the quantity of non-reducing sugars increased consistently throughout the growth period of the fruit. Reducing sugars tend to remain in higher quantity

than that of non-reducing sugars at all the stages except at the ripened stage, in which non-reducing sugars are two-fold higher than reducing sugars. According to Mazumdar and Majumder, starch is the major storage polysaccharide found in the fruits [19]. The amount of starch content was found to be 29.61 mg·100 g^{-1} at the young stage of the fruit, but during its growth and development the amount of starch decreased to 7.93 mg·100 g^{-1} at the ripened stage (*table II*). Thus, the results of the present study support the opinions of Mattoo *et al.* that starch is the main carbohydrate present in the fruits and, with the advancement of maturity, the accumulated starch is hydrolyzed into sugars, which is a characteristic event for the fruit ripening [20]. Besides, the results of the present study also support the view of Hulme, who noted that the sugar levels within the fruit tend to increase progressively at all successive stages of growth, development and ripening and opined that the increase in sugars may be mainly due to the hydrolysis of starch, which generally gets accumulated during the early stage of growth and with the onset of ripening [21] (*table II*).

Proteins are said to be the ubiquitous components of all living tissues. Although occurring in low concentration in fruits, they are involved in metabolism during growth, development and ripening of fruits. The amount of protein content in the khirni fruit in the present study is found to be more at its young stage (8.52 mg·g^{-1}) than that in its subsequent stages; 32% less at its premature stage (5.77 mg·g^{-1}). However, the level of protein quantity in mature khirni fruit rose to 7.84 mg·g^{-1}, but thereafter it remained more or less constant (*table II*). These results support the view of Hansen, who stated that proteins are intimately concerned with all physiological events including the synthesis and degradation of proteins [22]. The quantitative analysis of total free amino acids in the studied fruit showed that they decrease in their quantity by 44% from 7.06 mg·g^{-1} at the young stage to 3.99 mg·g^{-1} at the premature stage, but a significant increment in their quantity occurs at the ripened stage (8.12 mg·g^{-1}) (*table II*). Thus, the results of the present study support the observations of Frankel *et al.* who opined that the reduction in the quantity of total free amino acids may be due to their incorporation into proteins required for the synthesis of various ripening enzymes, and subsequently their utilization may also decrease, causing an increase in their quantity at the later stages [23].

Phenols, which are said to be important in determining the flavor and color of fruits, were found to be 1.26 mg·g^{-1} in the khirni fruit at its young stage, but they decreased to 0.90 mg·g^{-1} at its mature stage. However, eventually, the level of phenols in the ripened fruit increases significantly (4.28 mg·g^{-1}) (*table II*). Dilley, who reported a decreasing trend of phenols from high levels during early growth to low levels when the fruit attains maturity and thereafter becomes susceptible to the induction of ripening [24], reasoned that the biosynthetic mechanism of certain phenolic compounds appears to be responsive to environmental stimuli and certain phenolics are suspected of being involved in some types of stress responses.

Kidd and West divided fruits into two main categories: 'climacteric' and 'non-climacteric', according to changes in respiratory behavior [25]. Our study reveals that the rate of ethylene evolution in the fruit of *M. hexandra* was 0.132 µL·kg^{-1}·h^{-1} during the young stage but, subsequently, it decreased to 0.033 µL·kg^{-1}·h^{-1} at the preripened stage. However, with the onset of ripening the levels of ethylene evolution increased by 71%, reaching 0.112 µL·kg^{-1}·h^{-1} (*figure 2*). Although the rate of respiration decreases gradually from the young stage (0.034 µL·kg^{-1}·h^{-1}) to the premature stage (0.024 µL·kg^{-1}·h^{-1}), later it increases by 23% to 0.031 µL·kg^{-1}·h^{-1} at the mature stage (*figure 2*). Like other climacteric fruits, khirni fruit also demonstrates a climacteric rise in the rate of respiration and increase in ethylene production. Thus, the fruit of khirni falls under the category of climacteric fruits.

According to Stanley, amylase enzyme is known to hydrolyze starch [17]. During the course of our study, the specific activity of amylase was found to increase from the young stage (12.89 mg maltose released·min^{-1}·mg^{-1} protein) to the mature stage (14.36 mg maltose released·min^{-1}·mg^{-1} protein) but, in the preripened stage, it decreased to

Table II.
Changes in the biochemical composition of the fruit of *Manilkara hexandra* according to its sequential stages of growth and ripening. Values of means ± standard deviation followed by different letters are statistically significant according to Duncan's multiple range test (DMRT) at the 5% level (n = 3).

Stages of growth and ripening	Chlorophyll *a*	Chlorophyll *b*	Total chlorophylls	Total carotenoids	Total anthocyanins	Reducing sugars	Non-reducing sugars	Total sugars	Starch	Total free amino acids	Total proteins	Total phenols
			(mg·100 g^{-1})						(mg·g^{-1})			
Young	16.98 ± 1.08 d	8.68 ± 1.80 b	25.66 ± 0.96 c	4.15 ± 0.10 c	1.26 ± 0.20 a	5.95 ± 0.03 a	4.21 ± 0.45 b	10.17 ± 0.48 c	29.61 ± 1.36 e	7.06 ± 0.18 d	8.52 ± 0.14 e	1.26 ± 0.01 b
Premature	19.28 ± 0.65 e	19.14 ± 0.07 e	38.41 ± 0.72 e	4.11 ± 0.07 c	2.10 ± 0.09 c	8.73 ± 0.06 b	3.43 ± 0.14 a	12.17 ± 0.16 d	22.67 ± 0.19 d	3.99 ± 0.12 a	5.77 ± 0.16 b	1.74 ± 0.13 c
Mature	14.41 ± 0.11 c	13.64 ± 1.11 d	28.04 ± 0.99 d	5.08 ± 0.17 d	2.29 ± 0.06 d	9.17 ± 0.06 c	5.82 ± 0.43 c	14.99 ± 0.39 e	14.44 ± 0.71 c	5.76 ± 0.47 b	7.84 ± 0.36 d	0.90 ± 0.03 a
Preripened	2.13 ± 0.51 a	4.36 ± 0.14 a	6.49 ± 0.44 a	2.46 ± 0.44 b	1.45 ± 0.09 b	10.75 ± 0.04 d	9.24 ± 0.61 d	19.99 ± 0.56 b	9.68 ± 0.63 b	6.69 ± 0.11 c	7.67 ± 0.24 c	1.88 ± 0.11 d
Ripened	5.87 ± 0.35 b	13.30 ± 0.25 c	19.17 ± 0.20 b	1.56 ± 0.15 a	2.42 ± 0.13 e	11.63 ± 0.23 e	23.18 ± 0.88 e	34.81 ± 1.08 a	7.93 ± 0.73 a	8.12 ± 0.40 e	5.58 ± 0.25 a	4.28 ± 0.11 e

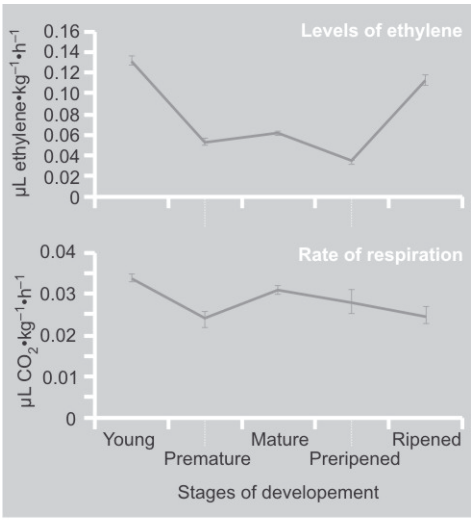

Figure 2.
Changes in the level of ethylene and rate of respiration during growth and ripening of *Manilkara hexandra* fruit.

2.36 mg maltose released·min^{-1}·mg^{-1} protein, while it more or less remained consistent until the ripened stage (*figure 3*). In contrast, a consistent and gradual increase in the specific activity of invertase was found throughout the course of growth and ripening of khirni fruit with 3.01 mg glucose released·min^{-1}·mg^{-1} protein at its premature stage; 4.56 mg glucose released·min^{-1}·mg^{-1} protein at the mature stage, and 4.87 mg glucose released·min^{-1}·mg^{-1} protein at the ripened stage (*figure 3*). It implies that during successive stages the enzyme invertase has an active role in conversion of sucrose into glucose and fructose.

According to Bowler *et al.*, the enzyme catalase is found predominantly in peroxisomes and also in glyoxysomes, where it functions chiefly to remove the H_2O_2 formed during photorespiration [26]. During the course of our study, the specific activity of catalase was found to decrease from 0.19 units·min^{-1}·mg^{-1} protein at the young stage to 0.09 units·min^{-1}·mg^{-1} protein at the preripened stage (*figure 3*). The specific activity of peroxidase enzyme was recorded to increase from 0.004 units·min^{-1}·mg^{-1} protein at the young fruit stage to 0.22 units·min^{-1}·mg^{-1} protein at its preripened fruit stage, which shows a remarkable increase of 55 times but, eventually (*i.e.*, during the ripening stage), it decreased to 0.16 units·min^{-1}·mg^{-1} protein (*figure 3*). Therefore, the results of our investigation are in agreement with the view of Jimenez *et al*. who stated that the antioxidant system, which includes catalase, superoxide dismutase, some peroxidase and many other enzymes, plays a crucial role in the ripening process [27].

According to Award and Young, enzymes that are involved in fruit cell wall metabolism during ripening include pectinmethylesterase (PME), polygalacturanase (PG), cellulase and β-galactosidase [28]. During the course of our study, the specific activity of PME was noted to be inconsistent with its varied levels: 0.02 A_{620}·min^{-1}·mg^{-1} protein at the young stage; 0.01 A_{620}·min^{-1}·mg^{-1} protein at the premature stage; 0.11 A_{620}·min^{-1}·mg^{-1} protein at the mature stage; 0.05 A_{620}·min^{-1}·mg^{-1} protein at the preripened stage; and 0.07 A_{620}·min^{-1}·mg^{-1} protein at the ripened stage (*figure 3*). However, the specific activity of cellulase enzyme of the currently studied fruit exhibits a steady and gradual increase in its specific activity (from 0.09 mg glucose released·min^{-1}·mg^{-1} protein at the mature stage to 0.26 mg glucose released·min^{-1}·mg^{-1} protein at the preripened stage), except in the ripened stage where it decreases significantly (0.08 mg glucose released·min^{-1}·mg^{-1} protein) (*figure 3*). In contrast, the specific activity of PG was found to be low until the mature stage [(0.06 and 0.03) mg glucose released·min^{-1}·mg^{-1} protein at the young and mature stages, respectively] but, subsequently, it increases [the preripened and ripened stages measuring (0.15 to 0.22) mg glucose released·min^{-1}·mg^{-1} protein, respectively) (*figure 3*).

From the above discussion it may be concluded that the underutilized fruits of khirni (*M. hexandra)* are very sweet [3], have high nutritional value [4], and the major physiological changes in relation to fruit ripening occur during its preripened and ripened stages. Thus, it is envisaged that the results of the present study would be useful in determining the maturity indices for harvesting of underutilized khirni fruit for its commercial exploitation.

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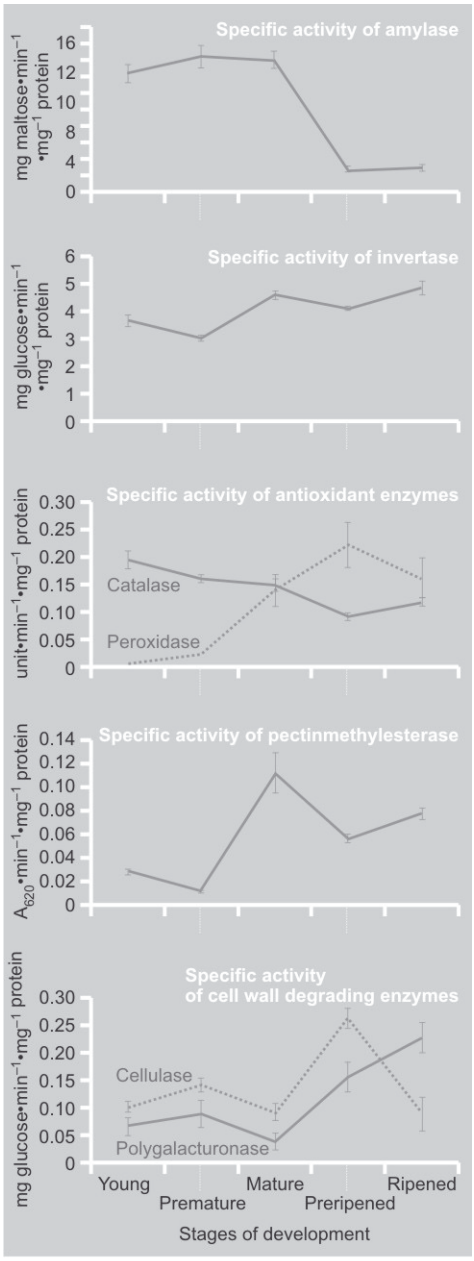

Figures 3.
Changes in specific activity of various enzymes involved during growth and ripening of *Manilkara hexandra* fruit.

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Cambios fisiológicos en el transcurso del crecimiento y de la maduración del fruto de *Manilkara hexandra.*

Resumen — Introducción. La maduración es un proceso que culmina en cambios de color, de sabor y de textura, que vuelven el fruto consumible. Debido a que intervienen en el desarrollo de un fruto maduro comestible una amplia serie de cambios fisiológicos, bioquímicos y organolépticos, estudiamos estos cambios en el fruto poco empleado, el khirni [*M. hexandra* (Roxb.) Dubard]. **Material y métodos**. Se analizaron en el fruto de *M. hexandra* en cinco estados de su desarrollo (joven, prematuro, maduro, a punto de madurez y en estado de madurez) los cambios de ciertos compuestos bioquímicos (clorofilos, carotenoides, antocianinas, azúcares, almidón, ácidos amínicos libres, fenoles, proteínas) así como la actividad específica de ciertas encimas (amilasa, invertasa, catalasa, peroxidasa, pectinmetilesterasa, poligalacturonasa y celulasa). **Resultados y discusión**. La pulpa del khirni tiene un sabor amargo durante su periodo de crecimiento, pero se vuelve azucarada cuando madura el fruto. Las clorofilas tienden a disminuir mientras que aumenta la cantidad total de carotenoides y de antocianinas. Asimismo se produce un aumento de la cantidad de azúcares, de proteínas y de fenoles según se acerca el estado de madurez, sin embargo, las cantidades de almidón y de ácidos amínicos libres totales tienden a disminuir. Por otro lado, el khirni muestra un comportamiento climatérico por el hecho de tener un índice creciente de su respiración y de su producción de etileno durante su maduración. Los cambios, entre moderados y significativos, de la actividad de encimas tales como la amilasa, la invertasa, la catalasa y la peroxdasa implicadas en un cierto número de reacciones catabólicas y anabólicas indican que estas encimas desempeñarían asimismo un papel activo en el curso del crecimiento y de la maduración del fruto de *M. hexandra*.

India / *Manilkara hexandra* / fruto / maduración / fisiología vegetal / crecimiento