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Developmental patterning and segregation of alkaloids in areca nut (seed of *Areca catechu*) revealed by magnetic resonance and mass spectrometry imaging

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ABSTRACT

Areca nut (seed of *Areca catechu*) is consumed by people from different parts of Asia, including India. The four major alkaloids present in areca nut are arecoline, arecaidine, guvacoline and guvacine. Upon cutting, the nut reveals two kinds of regions; white and brown. In our present study, we have monitored the formation of these two regions within the nut during maturation, using the non-invasive techniques of magnetic resonance imaging (MRI) and volume localized magnetic resonance spectroscopy (MRS). Electrospray ionization mass spectrometry (ESI MS) and desorption electrospray ionization mass spectrometry (DESI MS) imaging have been used to study the associated change in the alkaloid contents of these two regions during the growth of the nut. Our study reveals that white and brown regions start forming within the nut when the liquid within starts solidifying. At the final stage of maturity, arecoline, arecaidine and guvacoline get segregated in the brown region whereas guvacine gets to the white region of the nut. The transport of molecules with maturity and corresponding pattern formation are expected to be associated with a multitude of physiochemical changes.

1. Introduction

Areca nut or betel nut is the seed of *Areca catechu* L. (Arecaceae) (Fig. 1). It grows in the tropical climate of Asia, East Africa and the Pacific. In India, areca nut is associated with tradition and used in different cultural, social, and religious ceremonies. Besides, due to its inherent medicinal properties, it has found place in the traditional Indian medicinal system, *Ayurveda*, and is used for the treatment of leprosy, leucoderma, indigestion, etc., and is also used as a vermifuge. The use of areca nut dates back to the *Vedic* age (*ca.* 1500-500 BCE) and it has been mentioned in ancient writings. In different parts of Asia, it is consumed by people for its stimulatory action and it is a widely used masticatory (substance which increases the secretion of saliva when chewed). In India, it is traditionally consumed in the form of betel quid (a combination of betel leaf, areca nut, and lime) and people across the country consume it regardless of age, gender, and religion. Nowadays it is marketed in whole or as part of different products for direct consumption after different methods of processing. India, the largest areca nut producing country, accounts for 53% of the global production. Majority of areca nut grows in the Indian states of Karnataka and Kerala. Several small farmers and workers are involved in the cultivation of this crop. Employment and livelihood of many people in India are associated with the production of areca nut. As a consequence, areca nut cultivation has a direct impact on the society and economy of the country.

Areca nut largely contains sugars, lipids and polyphenols (Wetwitayaklung et al., 2006). It also contains a small amount of four structurally similar alkaloids: arecoline, arecaidine, guvacoline, and guvacine. Fig. 2 shows the molecular structures of four major alkaloids present in the nut. Polyphenols in areca nut comprise of condensed tannins, hydrolysable tannins, and flavonols. Polyphenols and alkaloids are the most bio-active components of areca nut. The former class of compounds are well known for their antioxidant property. They prevent oxidation of high density lipids (Han et al., 2007). Arecoline is the most abundant alkaloid in areca nut and its content in the nut increases during maturation, reaches a maximum, and then decreases drastically in the matured nut (Wang et al., 1997). In terms of biological activity, it is also more

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Fig. 1. Photograph of a nut-bearing areca nut tree. The inset shows a nut cut into two halves. It reveals W and B regions of the nut. At the earlier stages of growth, the contrast between these two regions is poor.

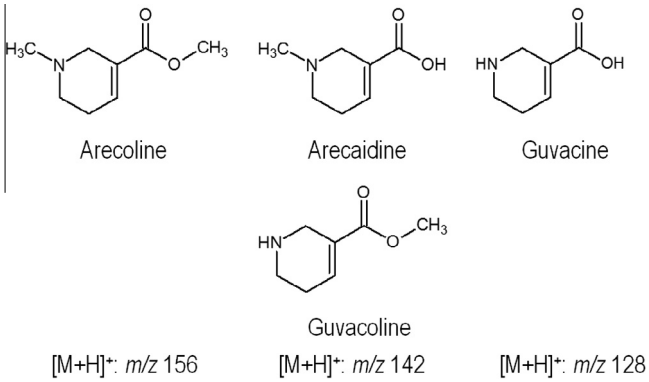

Fig. 2. Chemical structures of four major alkaloids present in areca nut.

potent than others. Arecoline is shown to be cytotoxic for human endothelial cells, and cell cycle is arrested upon exposure to arecoline (Ullah et al., 2014). Arecoline together with guvacoline reduce the survival of human buccal epithelial cells whereas arecaidine and guvacine have little effect on it (Sundqvist et al., 1989). Areca nut extract has been tested for its different biological activities. Aqueous extract of areca nut and arecoline is reported to have genotoxic effects (Dave et al., 1992). Extracts of several commercial products containing areca nut in different forms also show similar genotoxic effects (Polasa et al., 1993). Areca nut chewing or consumption of other related things like betel quid or different areca nut derived commercial products has been associated with the development of oral cancer. Several review articles present these aspects in detail (Adhikari and De, 2013; Jeng et al., 2001; Kumar, 2008; Mhaske et al., 2009; Nair et al., 2004; Zhang and Reichart, 2007). While the use of products related to areca nut is common in India, China, and a few other South Asian countries, the United States faces an increased risk of oral cancer cases related to the use of areca nut as well (Changrani and Gany, 2005), primarily owing to immigrant populations.

However, there are also several medicinal benefits due to areca nut. Its extract has been shown to have both anti-oxidative activity and free radical scavenging activity (Kim et al., 1997). Areca nut extract when supplemented with diet lowered the triglyceride absorption (Byun et al., 2001) as well as free cholesterol absorption (Park et al., 2002) in rats. It has also been shown to have antimicrobial activity (Hada et al., 1989). Comprehensive details regarding the medicinal use of areca nut can be found in review articles (Amudhan et al., 2012; Peng et al., 2015).

Upon cutting, areca nut reveals two regions: white (W) and brown (B) (Fig. 1 inset). The chemical composition of these two regions is unknown. In previous studies, areca nut was always considered as a whole nut rather than a mixture of two distinct regions. Spatially resolved aspects are rarely examined. In our study, we have focused on the formation of 'villi'-like structures in the nut while maturing and the chemical changes associated with it. MR imaging, volume localized NMR of fairly high resolution, and mass spectrometric techniques have been combined together for the study to get complementary information. Magnetic resonance imaging (MRI) was used to understand the evolution of villi-like structures in the areca nut while maturing, and electrospray ionization mass spectrometry (ESI MS) in conjunction with desorption electrospray ionization mass spectrometry (DESI MS) were used to understand the chemical changes associated with the maturity of the nut.

Earlier studies have shown how non-invasive MRI and MRS, as well as NMR spectroscopy can be used to understand plants. Examples include imaging of lipid distribution in the capsules of tobacco (Fuchs et al., 2013), imaging of water distribution in germinating tobacco seeds (Manz et al., 2005), post-harvest ripening of sweet lime (Banerjee et al., 2009), etc. Other applications of NMR in plant science can be found elsewhere (Borisjuk et al., 2012; Ishida et al., 2000). While MRI may be used to visualize internal structure and function non-invasively and non-destructively by mapping the distribution of selected molecular species (commonly water), it is perhaps not the ideal technique to deal with species that have very low concentrations. Although molecule specific chemical shift selective imaging or non-selective chemical shift imaging (CSI) may be performed to redress this, these techniques do suffer from poor sensitivity per unit measurement time owing to low metabolite concentrations. MR image guided volume localized spectroscopy, *ie*, MRS, on the other hand, provides the molecular fingerprint within chosen volume element(s) (voxel(s)) with sensitivity, *ie*, signal-to-noise-ratio, of the same order as in high resolution NMR. Mass spectrometry can also act as a complementary technique here to retrieve molecular information. Conventional mass spectrometry like ESI MS requires extraction of molecules from samples, losing spatial information. DESI MS, a mass spectrometric tool which works under ambient conditions without sample preparation or extraction (Monge et al., 2013), was introduced in 2004 (Takats et al., 2004) and is capable of imaging molecular species, and sampling is done from the surface, not from the interior. Due to its excellent compatibility in working with biological samples, it has gained the attention of researchers in the field of plant science and other biological sciences. It was used for rapid identification of anti-cancer molecules from plants (Srimany et al., 2011),

study of the distribution of metabolites in different parts of plants like seeds (Ifa et al., 2011; Mohana Kumara et al., 2015), leaves (Muller et al., 2011; Thunig et al., 2011), petals (Thunig et al., 2011), fruits (Cabral et al., 2013), etc.

2. Results and discussion

2.1. Probing areca nut by MRI and MRS

During maturation, an areca nut goes through several transformations. Transformation from the initial liquid stage to a patterned hard nut consisting of W and B regions is associated with different physical and chemical changes. To understand the structural changes during maturation we have chosen to study areca nut at six maturity stages; we discuss below in detail three significantly different maturity stages, while some information on the other stages is included in Supplementary material. We have analyzed multiple samples, typically 3, at the same maturity stage for statistical validity of our data, especially at the initial stages. Stage 1 is a tender areca nut, stage 2 is a ripened nut, and stage 3 is a completely matured nut without husk. Structural evolution in the nut was studied by MRI. As MRI is a non-invasive technique and can generate layer by layer images of the whole sample, it is best suited for our purpose. Fig. 3 displays typical MR images at three different stages of maturation. Supplementing this, the volume localized NMR spectrum gives *in situ* information on the metabolite soup, *ie*, mixture of various metabolites, at the molecular level, from the chosen region within the sample, the region of interest being selected with the help of the MR image. Fig. 4 shows typical

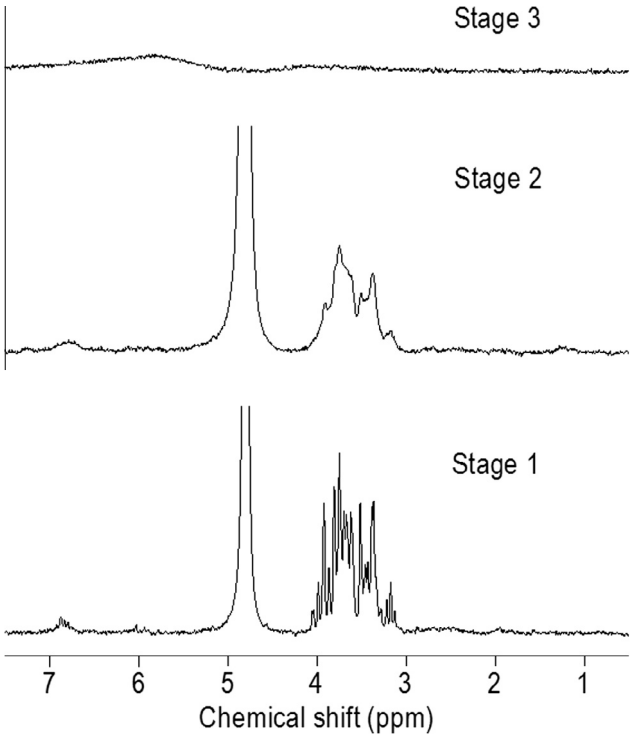

Fig. 4. Volume localized ^{1}H NMR spectra of areca nut samples of three different maturity stages.

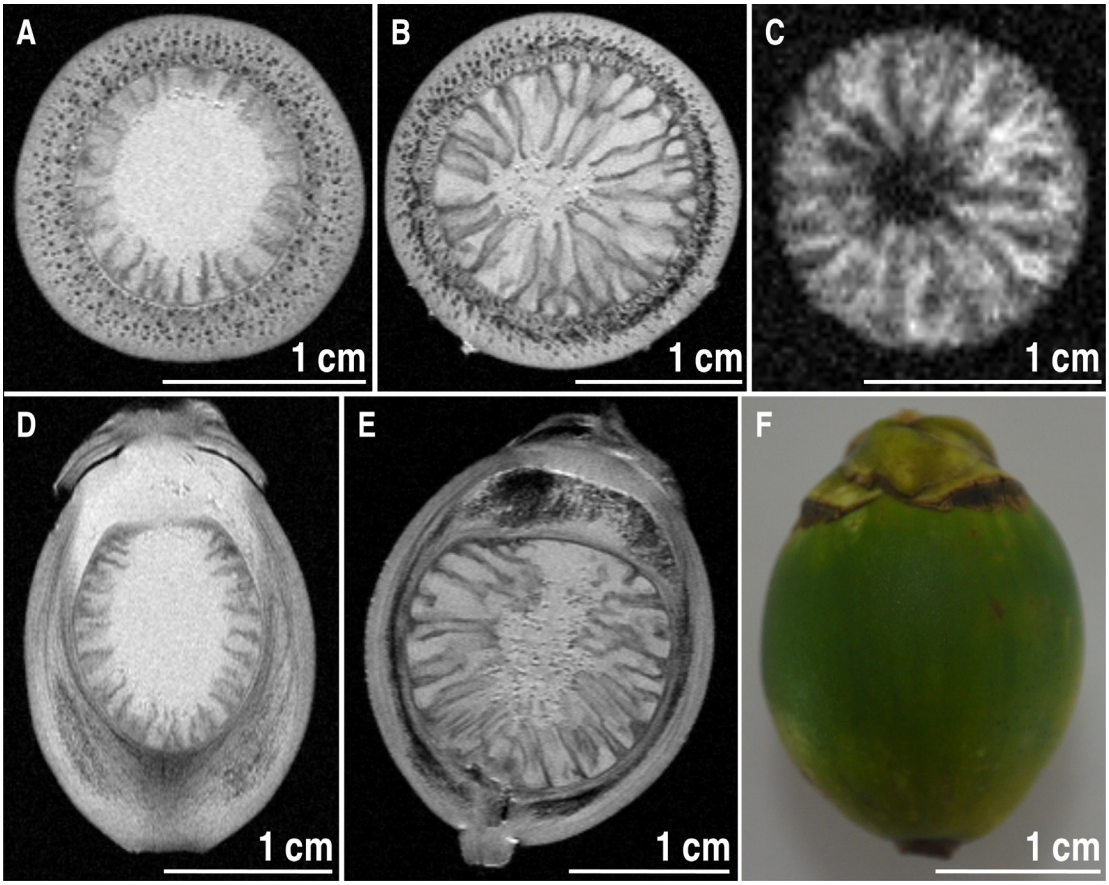

Fig. 3. MRI images of areca nut samples of three different maturity stages. Axial view: (A) stage 1 sample, (B) stage 2 sample, (C) stage 3 sample. Coronal view: (D) stage 1 sample, and (E) stage 2 sample. (F) Photographic image of a stage 2 areca nut sample.

volume localized NMR spectra of areca nut at three different maturity stages. Volume localized spectra were collected from different nuts at different maturity stages, grown in a cluster of plants in the same farm. We have performed the PRESS experiment with a single voxel selection from a particular nut, repeated however over typically 3 samples at the same maturity stage. The typical voxel position is shown in Fig. S1. As for the voxel co-ordinates, the voxel position is not identical for different maturity stages, as the fruit matures and its size changes. Voxels were chosen so as to avoid the husk and the center pith region, while still being as close to the resonator center and field center as possible, in order to ensure good rf and magnetic field homogeneity. To observe molecular species with millimolar concentration in the presence of a high concentration of water protons (~75–100 M), it is necessary to employ water suppression techniques in volume localized NMR. The residual water peak, appearing at 4.8 ppm, is used as the chemical shift reference. Due to the liquid nature of areca nut matrix in the initial stages of development, fairly good spectral resolution is observed in the volume localized ^{1}H NMR spectrum of stage 1 sample. As the fruit matures, water content and mobility in the sample reduces as seen in the MRI images (Fig. 3), and the fruit gradually develops into a hard nut. Concomitantly, a progressive degradation of resolution is observed in the spectrum, attributable to reduced molecular mobility. Various molecular species in areca nut include sugars, polyphenols, lipids, and alkaloids (Wetwitayaklung et al., 2006; Yenrina et al., 2014; Zhang et al., 2014, 2010). Although earlier studies differ from each other on the exact concentrations of these molecules, the concentration of sugars is reported to be two orders of magnitude higher than that of the alkaloids (Wetwitayaklung et al., 2006; Yenrina et al., 2014). Peaks observed in the 3.1–4.1 ppm region correspond to the sugar protons. Olefinic proton peaks from four different alkaloids present in areca nut are expected to appear in the 6.8–7 ppm region whereas aliphatic ring protons should appear in the 2–3 ppm region. Chemical shift information is best discerned from the spectrum of the sample at the pre-tender stage (as shown in Fig. S2). However, for this liquid-like sample, no interesting image information is to be gleaned, such as the formation of villi. Although the signal intensity varied slightly from sample to sample for the same stage of growth, we have observed proton resonances in these regions as anticipated. Peaks around 2–3 ppm appeared to be much broader than the sugar proton peaks. Further, lipid content seems to increase considerably as the fruit matures (*ca.* 1.5 ppm). Volume localized spectra at different stages of maturation are shown in the Supplementary material, including the pre-tender stage of areca nut, as well as at five further stages of maturation as a stacked plot; a spectrum with inset depicting the typical spectral voxel is also shown, as noted earlier.

More insight into the structural evolution inside the shell of areca nut is obtained from MRI images that were taken from a set of parallel slices in three orthogonal planes of the samples. Fig. 3 shows representative MRI images of three different stages of the samples with the axial view (stages 1, 2, and 3) and coronal view (stages 1 and 2), as well as a photographic image of stage 2 sample. Typical in-plane MRI image resolution is 156 μm × 156 μm. It is evident from Fig. 3A and D that in the initial stage of maturity, the core was liquid, which started solidifying inwards from the peripheral region. During this solidification process itself, the B and W regions got separated. It should also be noted that the top portion of the nut (which is attached to the tree), does not show any solidification (Fig. 3D). This end of the sample was excluded from solidification and vilii-like structure formed at the early stage. As the nut became more matured (stage 2 sample), solidification process as well as patterned structure formation increased and the patterns reached closer to the center (Fig. 3B and E). At this point of maturity, the central portion also got solidified but did not exhibit any patterned structure in this region. The top portion which is attached to the plant got solidified at this stage without forming any patterned structure either (Fig. 3E). From this point on, no further pattern formation occurred; but the nut became harder. At stage 3, the nut matured completely and became a solid but no new B region was formed in the center (Fig. 3C). The fact that during the maturation process, the water concentration and mobility reduced was reflected in the MRI images and the stage 3 sample gave poorer data quality for this reason, requiring larger slice thickness. From the images of all three different stages, we can say that patterned structure formation in the nut occurs in the early stages of maturity, when the liquid inside the nut gets solidified. This process continues to some extent and at some point of time during the later stages of maturity, patterned structure formation stops and only solidification continues till it becomes hard, similar to wood. In the whole process, the top region of the nut which was originally attached to the plant remains free of any patterned structure.

Since the concentration of the alkaloids is very small in comparison to other molecular species, *eg.*, sugars, and also due to the loss of spectral resolution at the final stages of growth, it was difficult to track the spatial changes in alkaloid concentration as a function of maturation by non-invasive volume localized NMR, especially at relatively low field strengths such as 4.7 T. MRS experiments at a higher field such as 11.7 T would substantially help in this regard, with a resonator that accommodates the whole areca nut, although the thin B region would probably still not be amenable for definition of a separate voxel. High resolution NMR on fruit extract could be an alternative approach.

2.2. Alkaloids observed in different stages of areca nut and their distribution in brown and white regions – ESI MS and DESI MSI analysis

Mass spectrometry is yet another technique to track the molecular changes as a function of fruit ripening. To this end, two different mass spectrometric techniques were used to get molecular and spatial information. In this work, we have focused mostly on four structurally related alkaloids (arecoline, arecaidine, guvacoline, and guvacine) present in the nut and tried to understand their spatial and temporal evolution. In the subsequent experiments with mass spectrometry, we have used nuts of similar maturity stages as used in earlier experiments with MRI and MRS.

When stage 2 areca nut was cut, it revealed both W and B regions. At this stage, these regions were not completely solidified. Both the W and B regions were separated by cutting with a razor blade and the compounds were extracted in methanol. For stage 3 areca nut, flakes were made from the whole nut and then W region was separated by cutting with a razor blade and the compounds were extracted. In this case, due to the very thin B region, separating them from the W region was not possible. For this reason, to get the molecules from the B region, whole flakes were taken for extraction. They contained both W and B regions. Stage 1 sample due to its liquid-like nature have been excluded from mass spectrometric studies as no spatial information could be obtained from it. However, it was used for magnetic resonance studies to obtain structural information, primarily.

The methanol extracts were infused for ESI MS measurements and the experiments were performed in triplicate with 3 different nuts of same maturity stages. Fig. 5 shows typical ESI spectra of W and B regions of stage 2 areca nut. For the W region sample, in the low mass range, three peaks appeared at *m/z* 156, 142, and 128. The peak at *m/z* 156 corresponds to protonated arecoline, *m/z* 142 corresponds to both protonated arecaidine and guvacoline, and *m/z* 128 corresponds to protonated guvacine. The confirmation of these compounds was achieved by tandem mass spectrometry using CID and the fragmentation patterns are shown in Figs. S4–S7.

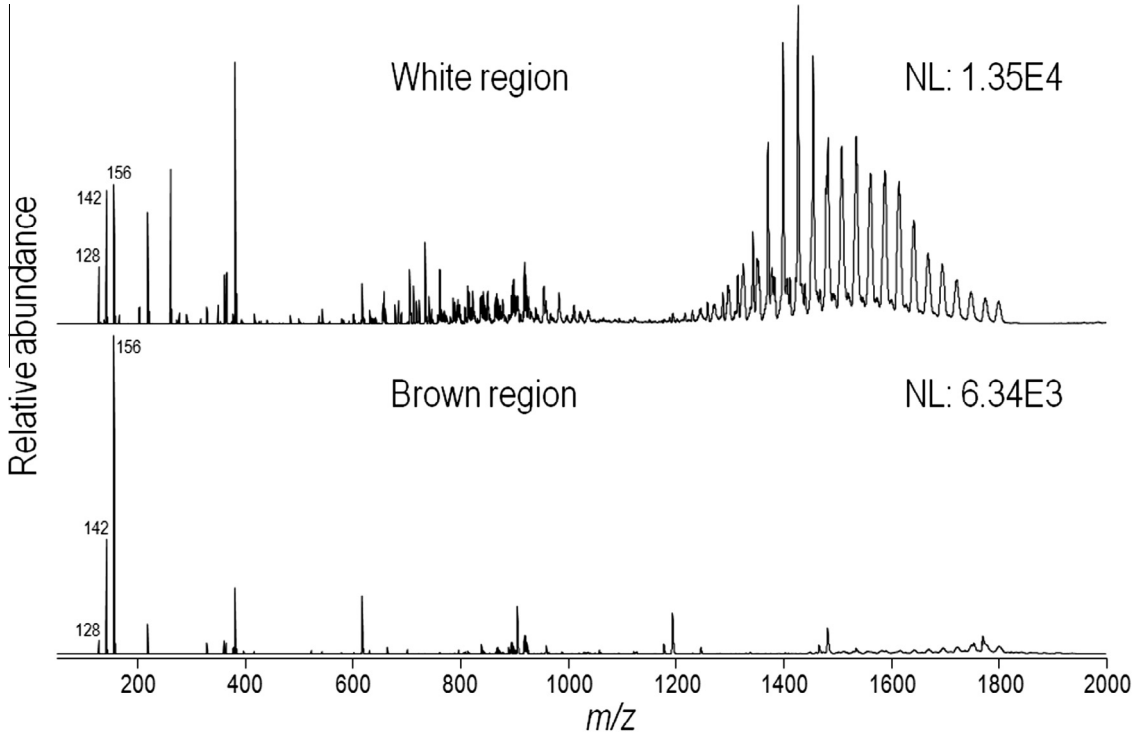

Fig. 5. ESI MS spectra of methanol extracts from W and B regions of stage 2 areca nut.

From ESI MS measurements, it was not possible to distinguish between arecaidine and guvacoline as both of them have the same mass and gave peak at *m/z* 142 although their presence was confirmed by tandem ESI MS. For that reason, in the subsequent discussion, we have always considered the peak at *m/z* 142 as a combination of both arecaidine and guvacoline. Besides these, there were several other peaks in the spectrum. For the B region of the sample, the same peaks at *m/z* 156, 142, and 128 appeared in the spectrum. There were several peaks as well in other regions of the spectrum and the notable difference between the B and the W regions was in the high mass range where some peaks corresponding to polymeric compounds appeared only for the W region. Fig. 6 shows typical ESI spectra of stage 3 sample. For W region, in the low mass range, there was a peak at *m/z* 128 while peaks at *m/z* 156 and 142 were not seen. For the combined sample composed of W and B regions, in the low mass range, all three peaks at *m/z* 156, 142, and 128 appeared. In both the samples, several other peaks were observed in the spectra. Here, the polymeric peaks of high mass

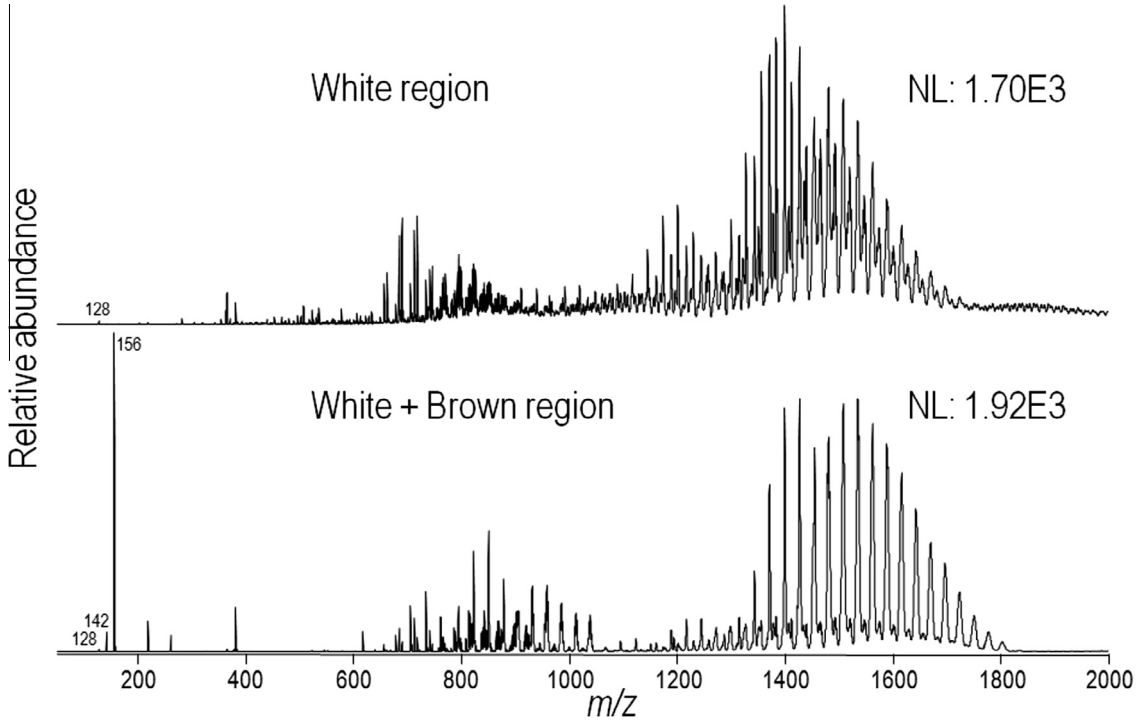

Fig. 6. ESI MS spectra of methanol extracts from W and combined W and B regions of stage 3 areca nut.

range appeared in both the samples as both of them contained W regions.

If we now take a closer look at all the ESI MS data in the low mass range for the four major alkaloids, several things can be understood regarding their distribution during maturity of areca nut. W region of stage 2 sample contains all the four major alkaloids and the abundance of arecoline is almost similar to the combined abundance of arecaidine and guvacoline. Guvacine is present in low abundance. For the B region of the same sample, arecoline is present in very high abundance compared to the others. Once again, the abundance of guvacine is low here as well. When we go to the stage 3 sample, the W region contains only guvacine, other alkaloids are absent here. In the B region, arecoline, arecaidine, and guvacoline are present but the presence of guvacine cannot be confirmed as the sample used here is a combination of both W and B regions and the W region already contains guvacine. Whatever may be the case with guvacine, the relative abundance of arecoline in the B region of stage 3 areca nut is high compared to the other alkaloids.

As ESI MS experiments were not able to provide spatial information about W and B regions of stage 3 areca nut sample, we did DESI MS imaging experiment on the stage 3 sample. The cross-sectioned areca nut of stage 3 was imaged by DESI MS and Fig. 7 shows a photographic image as well as mass spectrometric images of different ions from the same sample. A representative average DESI MS spectrum of the sample (combination of both W and B regions) obtained from imaging experiment is also shown in Fig. 7. Please note that the peak at *m/z* 128 is almost in the noise region and it is due to the fact that the spectrum is an average one over both the W and B regions. Fig. 7B and C show the distribution of *m/z* 156 and 142, respectively. It is clear from Fig. 7B and C that arecoline, arecaidine, and guvacoline are present preferentially in the B region of the nut while these alkaloids are present to a limited extent in the W region. Fig. 7D shows the distribution of *m/z* 128 and it is evident that guvacine is present preferentially in the W region and only to a very small extent in the B region.

We can conclude from all the mass spectrometric data that in the ripened areca nut, arecoline, arecaidine, and guvacoline get segregated in the B region and guvacine exists in the W region. In an earlier study, it was shown that the concentrations of all the four major alkaloids (arecoline, arecaidine, guvacoline, and guvacine) decrease from the unripe nut to the ripened nut (Wang et al., 1997). In that study, the nut was considered as a whole entity and no differentiation was made between the W and B regions. In the context of our present study and with the input from the earlier study, we can say that individual alkaloid content decreases in ripened areca nut and higher amount of arecoline together with lower amount of arecaidine and guvacoline preferentially stay in the B region while further lower amount of guvacine preferentially stays in the W region of the ripened areca nut.

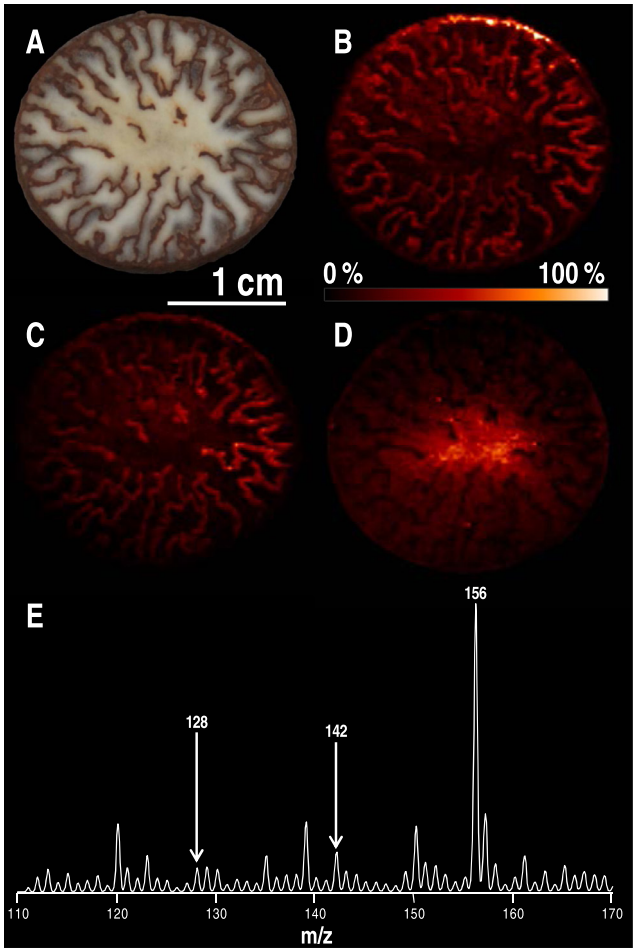

Fig. 7. (A) Photographic image of cross-sectioned stage 3 areca nut and its DESI MS images showing the distribution of (B) *m/z* 156, (C) *m/z* 142, and (D) *m/z* 128. (E) Representative average DESI MS spectrum of stage 3 areca nut sample (combination of both W and B regions) in zoomed view. Scale bar of 1 cm is applied to all the images. Intensities are color coded in B, C, and D.

2.3. Plausible mechanism of areca nut maturation: pattern formation and alkaloids segregation

Based on the results obtained, we speculate a mechanism for the growth of structured patterns in areca nut. As noticed in the MRI images (Fig. 3D and E), the top portion of the nut which is connected to the tree does not get solidified during the initial stage of growth. It is probably to make way to pump in several resources into the nut. At a later stage of growth, the same portion may be used to pump out resources like alkaloids from the nut as the valuable resources needed for the plant are recovered. From the mass spectrometric data it is evident that the W region in the ripened nut (stage 3) contains only guvacine, not other alkaloids which have additional functionalization. Note that all the other alkaloids have functionalization at the nitrogen or carboxyl ends which make them less polar. One probable reason for this preferential retention is that the W region, due to its hydrophilic nature, retains the more polar molecule, in this case, guvacine. Size may also play an important role here and helps to expel bigger molecules more efficiently during the solidification process. During the early stages of solidification, the transportation of alkaloids is possible due to the semi-solid nature of the mass formed. But at the final stages of solidification, transportation of alkaloids becomes difficult and some of the alkaloids cannot go out completely and remain in the nut. That is why the final stage of the nut (stage 3) also contains some alkaloids which are concentrated in the B region. There also exists the possibility of interconversion of these four alkaloids, owing to their structural similarities, during maturation of the nut. Because of the complexity of a real biological system, it is very difficult to assess the mechanism with certainty. The present work is an attempt to give a glimpse into the phenomenon of "*maturation of areca nut*" and we believe that understanding of the dynamics of the events of molecular transport leading to patterned structures, in conjunction with polymerization and solidification require theoretical modeling. Local concentration of each of the species must be analyzed as a function of time, as also the variation in the chemical structures with time. Patterned structure formation can be fully understood only with these inputs.

3. Conclusions

In the present work, we have shown by MRI that during maturation of areca nut, the liquid content within starts solidifying from the peripheral regions of the nut and solidification process moves towards the center. Volume localized MRS affords fairly high resolution molecular fingerprinting especially at the early stages, and demonstrates the presence of sugars, lipids, and alkaloids, although on commencement of structure formation, the present voxel resolution does not permit differentiation of different zones of the structure in the moderately tender nut. During the solidification process itself, B and W regions start forming. Furthermore, by different mass spectrometric methods, we have also shown that in the ripened areca nut, arecoline, arecaidine, and guvacoline get segregated in the B region while guvacine gets segregated in the W region. As the major fraction of the alkaloids stay in the B region of the ripened areca nut, it is safer to consume the W portion of the ripened nut. Thus areca nut can be used in a safer way to tap its medicinal activities if the W and B regions are separated.

4. Materials and methods

4.1. Materials and reagents

Different stages of areca nut were collected from the farm of Mr. Ravi V. Bhat, located in the state of Karnataka, India. Nuts were plucked at different maturity stages from a cluster of plants grown at the same farm, and were stored at low temperature to prevent any postharvest changes until the experiments were performed in the laboratory. For ESI MS and DESI MS measurements, HPLC grade methanol from Sigma–Aldrich was used.

4.2. MRI and MRS measurements

All the experiments were performed on a 200 MHz 47/40 Bruker BIOSPEC MRI system using a 7.2 cm $^{1}H/^{31}P$ resonator. Multi-slice multi echo images were acquired with the 2D spin echo imaging sequence. For stage 1 and stage 2 samples, images were collected from slices of 1 mm thickness. However, for the sample at stage 3, a slice thickness of 2 mm was used to improve the signal-to-noise-ratio. 2D images were collected from sets of three orthogonal imaging planes. Volume localized NMR was used to monitor the molecular level changes at different stages of growth of areca nut. To this end, point resolved spectroscopy (PRESS) with water suppression (VAPOR with bandwidth 150 Hz) was used with the following parameters: voxel size: $4 \times 4 \times 4\ mm^3$, number of scans: 256, echo time: 13.408 ms, and repetition time: 2.5 s.

4.3. ESI MS measurements

For ESI MS measurements, a Thermo Scientific LTQ XL mass spectrometer was used. Different portions of areca nut were soaked in methanol overnight (approximately for 12 h) and the resultant extracts were collected, centrifuged, and used for further ESI MS measurements. The extracts were infused with a flow rate of 5 μL/min and 5 kV spray voltage was used to collect data in positive ionization mode. Tandem mass spectrometric measurements were done with collision induced dissociation (CID).

4.4. DESI MS measurements

For DESI MS measurements, a 2D DESI source from Prosolia was used. It was attached with a Thermo Scientific LTQ XL mass spectrometer. A ripened areca nut was cut in a disk-like shape of about 4 mm thickness and fixed on a glass slide with double sided tape. The glass slide was then mounted on the DESI stage capable of moving in XY-plane. Methanol was used as spray solvent in DESI MS experiments and it was sprayed with the assistance of 150 psi pressure of nebulizer gas (N_2). DESI spray emitter to surface distance was maintained at 2 mm while the emitter to capillary inlet distance was 3 mm. Other parameters like solvent flow rate 5 μL/min, spray voltage 5 kV, capillary temperature 250 °C, capillary voltage 45 V, and tube lens voltage 100 V were maintained throughout the experiments. Data were collected in positive ionization mode. A pixel size of 250 μm × 250 μm was used to image a sample area of 30 mm × 25 mm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2016.02.002.

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