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# BARLEY STARCH GRANULE DEVELOPMENT[1]

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## 1. INTRODUCTION

Starch, the main constituent of barley grains (60-64% of kernel dry weight), exists in the form of semi-crystalline granules deposited in kernels, stems, and leaves as reserve food supply for periods of dormancy, germination, and growth (*10*). In kernels, starch is deposited in the endosperm, where it is synthesized and stored in amyloplasts, a sub cellular organelle (*6, 7*).

Several sizing techniques such as microsieving, Coulter Counter analysis, and digital image analysis (*8, 11, 13*) have been used to measure barley starch granule size, and typically have indicated a bimodal distribution for most genotypes. The two widely acknowledged classes of granules composing the bimodal distribution are: large, type A, and small, type B (*5*).

The mechanism by which A and B granules are initiated is still being investigated (*14*) and little detailed information is available on the biosynthesis of starch granules. Studies on barley starch granule accumulation have shown that A granules are initiated during endosperm cell division, 12-18 days after anthesis, and increase in size throughout

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grain filling (*4*), while B granules are laid down approximately 14-19 days after anthesis.

A study on developing wheat (*3*) showed that starch granules initiated during the first 4 days after anthesis (A type) enlarge to their maximum diameter as early as 19 days after anthesis while B granules, initiated at 10 days after anthesis, began to enlarge only at 21 days after anthesis, reaching maximum diameter at 35 days after it.

This study was conducted to learn whether size distribution of barley starch granules changes during kernel development.

## 2. MATERIALS AND METHODS

The effect of kernel development stage on starch granule traits was studied in three cultivars: Excel, a new American six-row malting cultivar; Condor, a Canadian two-row feed barley, and Alexis, an European two-row malting variety. The growing conditions at Crookston-MN, USA in 1992 were characterized by a cool summer and ample moisture which resulted in an excellent crop with plump kernels. Alexis and Condor were similar in days to heading and about one week later than Excel. Harvest ripe stage occurred about 40 DAH, i.e. about 6 days following the last sampling date. A split-plot in time design with three replications was used. Cultivars constituted main plots and kernel development stages subplots. Four stages of kernel development, 13, 20, 27, and 34 days after heading (DAH) were sampled.

Following harvest, spikes were dried at 37°C for 24 hours and then stored until required for starch granule measurements. A sample of 10 g of seed of each genotype from each experimental unit was ground on a Brinkmann sample mill (Retsch), using a 0.5 mm screen. One-half gram of the resulting flour was diluted in 5 ml sodium dodecyl sulfate (2% w/v) to remove crude protein, and was brought into suspension by vortexing for 60 seconds. The suspension was then submitted to two minutes of ultrasonication (Bransonic 2200). To stain the starch granules, a few drops of iodine solution were added to the suspension and the sample vortexed for 60 seconds. After the samples were stained with iodine and before each sample was analyzed they were vortexed vigorously for 15 seconds to obtain a uniform homogenate representing the suspension. This step was important because small granules remain in suspension for a longer period than large granules (2). Next, 50 µl of suspension was removed with aid of a pipette, placed on a 75 x 25 mm microscope slide and covered with a 18-mm square coverslip. For each experimental unit (subplot) seven to ten microscopic fields in each slide were measured. Thus a minimum of 600 starch granules were measured per experimental unit.

Starch granule size was measured by digital image analysis (DIA), using IBAS 2.0 software on a Kontron image analyzer (Kontron Elektronic Gmbh, Germany), interfaced with a Fischer microscope through a video camera (Sony CCD, model XC-77), for data collection.

At early stages of kernel development small granules consist of a blend of immature A and B granules (*9*). In part one of this paper, granules larger than 10 µm in diameter were labeled "large granules" (L) while granules with diameter less than 10 µm were designated "small granules" (S).

Using DIA, starch granule images were captured using a green filter to enhance contrast. The starch granules stained dark purple, and a sharp focus was obtained for a crisp gray digitized image that was displayed on a high resolution monitor. A 40x objective was used for all readings.

Each particle in the final field image was identified and area, maximum-, minimum- and equivalent diameter, perimeter, and circularity shape factor were measured for each discriminated starch granule. It should be recognized that a two-dimensional image was measured by the DIA, and therefore the dimensions, specially of large granules, refer to those lying flat. From those traits, surface area and volume for large and small granules, ratio of number of small to large granules and proportion of large granules to total starch by volume were derived to more fully characterize the starch granules.

L-granule dimensions were estimated assuming an oblate-spheroid shape and S-granule dimensions were calculated assuming a spherical shape (*1*). To calculate L granule surface area and volume, their eccentricity (e), which is an indication of the sphericity of the granule, was needed. It was calculated as $e = 2c/MD_L$, where c is the hypotenuse of a right triangle and $MD_L$ is maximum diameter of the granule (*1*). It was also necessary to estimate granule thickness (T) to compute c. By preparing slides crowded with granules, sufficient granules were found lying "sideways" which permitted measurement of T. One-hundred minimum diameter values were averaged to obtain the mean thickness (T). Mean thickness obtained from the measurements and used in this study was T = 4.23 µm.

The six starch granule traits reported here were calculated as described below. Component terms are defined following the formulas.

*Surface Area of a Granule (S)*: Outer surface area expressed in µm$^2$, was calculated as previously proposed (*1*):

*L granules*: $$SL = \frac{\pi}{2} MD_L^{2} + \pi \frac{T^2}{\varepsilon} \ln \frac{(1+\varepsilon)}{1-\varepsilon}$$

*S granules*: $SS = \pi ED^2$

*Granule Volume (V)*: Granule volume expressed in $\mu m^3$ was calculated as:

*L granules*: $VL = \frac{\pi T}{3} MD_L^2$

*S granules*: $VS = \frac{\pi ED^3}{6}$

*Proportion of L granules to Total Starch (PV)*: The cumulative volume of L granules in relation to total volume of granules, expressed in %, was calculated as:

$$PV = 100\, V_L/(V_L + (V_S \text{ Ratio S/L})$$

*Ratio of Number of S to L Granules (Ratio No. S/L)*: Ratio of number of S to L granules was calculated as:

$$\text{Ratio No. S/L: } \frac{\text{Number of S}}{\text{Number of L}}$$

In the above equations $MD_L$ is maximum diameter of an L granule, ED is equivalent diameter of a granule, ln is the natural logarithm, V is volume of granule, $\varepsilon$ is eccentricity, and T is thickness of the granule, which was found to be on average 4.23 μm.

Using SAS, analyses of variance were performed on granule means for all traits. Statistical significance was evaluated using analysis of variance F tests.

Changes in starch granule size distribution during kernel development were also followed by scanning electron microscopy for the cultivar Alexis. Ten milligrams of flour was attached to stubs by means of double-sided sticky tape, then coated with carbon. The mounted samples were examined in a Hitachi scanning electron microscope, model S 450 at an accelerating potential of 10 KV. Micrographs were taken on Polaroid type 55 P/N film.

## 3. RESULTS AND DISCUSSION

Statistically significant differences among the four stages of development were found for all granule traits (Table 1), suggesting that starch granule traits changed during development, i.e. from 10 to 34 DAH. The genotype by development stage interaction was significant for surface area and volume of large and small granules, indicating that genotypes had different patterns of granule development.

Starch granule means and standard deviations for the four stages of kernel development in three cultivars are presented in Table 2. Surface area and volume of large granules increased over time for all cultivars; however, the degree of enlargement was somewhat different for each cultivar, as indicated by the significant genotype by stage of development interaction. On average, large-granule surface area of Alexis and Excel increased 21% from 10 to 34 DAH, while large-granule surface area of Condor increased 29% in the same period.

Surface area and volume of small granules decreased over time in the three cultivars. Small granules of Alexis showed the largest reduction, 73% from 10 to 34 DAH, while Condor and Excel were similar, 55% and 58% reduction in the same period, respectively (Table 2).

Proportion of large granules to total starch for each cultivar consistently increased over time. Alexis and Excel moved to the 80% level of proportion of large granules to total starch by 20 DAH, while Condor reached 80% by 27 DAH. By 34 DAH all cultivars had reached more than about 94% of proportion of large granules to total starch (Table 2).

While significant on some cases, genotype by stage of development interaction was not large enough to cause rank changes for any of the granule traits (Table 2). Thus, we considered it appropriate to use the three cultivar mean data for discussion of general trends.

Changes for six starch granule traits pooled for three cultivars over the four stages of development are given in Figures 1 e 2. Large starch granules increased in size during kernel development, as shown by the increase in surface area and volume of large granules during the 21 day period. The increase in surface area and volume observed for the large granules is accounted for by growth of granules during the process of kernel development and ripening.

Surface area and volume of small granules decreased over time in the three cultivars. Two factors may have contributed to that reduction. The first was shifting of larger "small granules", presumably immature A granules, into the "large granule" class, due to growth. The second was the formation of new truly-small granules, which are of tiny size in the early development stages. BECHTEL et al. (*3*), working with wheat, indicated that a flush of small granules occurred around 14 days after anthesis and that they did not enlarge in the following 10 days. Newly formed barley

TABLE 1 - Mean squares for six starch granule traits from the analyses of variance for four stages of kernel development and three varieties, grown in Crookston, 1992

| | | Surf. area | | Volume | | PV[1] | Ratio |
|---|---|---|---|---|---|---|---|
| Source | df | L | S | L | S | | No. S/L |
| Replication | 2 | 1916.03 | 74.32 | 10163.37 | 20.68 | 176.72 | 26.00 |
| Genotype | 2 | $3387.05^{**}$ | 50.38 | $18091.30^{**}$ | 43.61 | $244.24^{**}$ | 142.30 |
| Error A | 4 | 2531.61 | 76.91 | 13624.20 | 49.18 | 78.99 | 24.78 |
| Stage | 3 | $2218.84^{**}$ | $1057.58^{**}$ | $11807.32^{**}$ | $617.68^{**}$ | $182.24^{**}$ | $592.81^{**}$ |
| Genotype*Stage | 6 | $713.32^{*}$ | $119.63^{*}$ | $3884.24^{*}$ | $87.23^{*}$ | 32.92 | 87.40 |
| Error B | 18 | 255.25 | 35.89 | 1363.54 | 26.02 | 35.01 | 97.07 |

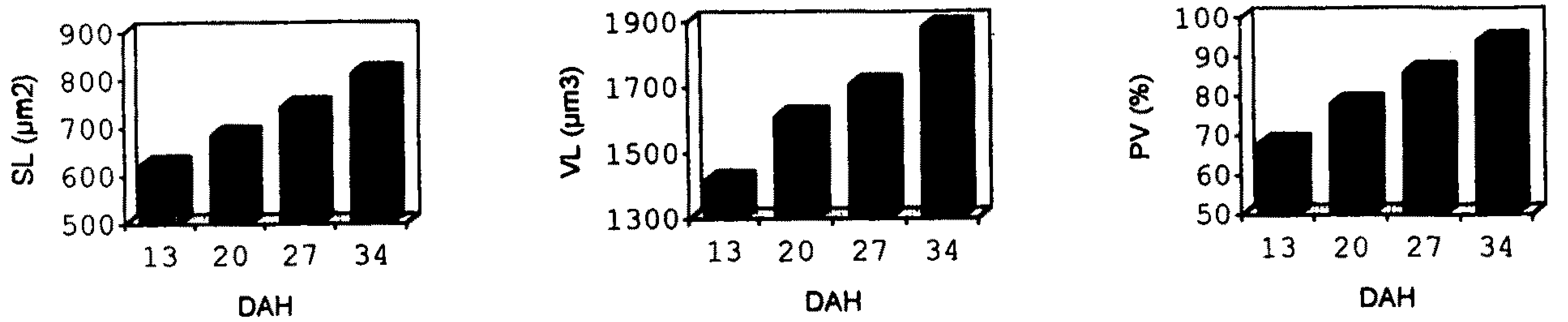
$^{**}$, $^{*}$ Significantly different at 1% and 5% probability level respectively

[1] PV, Proportion of L granules to total starch by volume

TABLE 2 - Means and standard deviations of six starch granule traits for four stages of kernel development and three varieties grown at Crookston, MN, 1992

| Days after heading | Surface area |  | Volume |  | PV[1] | Ratio |
|---|---|---|---|---|---|---|
|  | L | S | L | S |  | No. S/L |
|  | ($\mu m^2$) |  | ($\mu m^3$) |  | (%) |  |
|  |  |  | **ALEXIS** |  |  |  |
| **13** | 629±11 | 78.0±6.5 | 1431±21 | 68.8±5.4 | 62.3±5.0 | 22.29±3.71 |
| **20** | 681±13 | 26.4±2.1 | 1704±26 | 14.2±1.8 | 82.1±1.5 | 38.19±4.77 |
| **27** | 776±12 | 25.0±1.0 | 1799±23 | 13.9±0.8 | 85.5±2.3 | 20.82±5.63 |
| **34** | 890±16 | 21.9±1.5 | 2097±31 | 10.2±1.0 | 94.1±2.6 | 9.13±2.21 |
|  |  |  | **CONDOR** |  |  |  |
| **13** | 588±9 | 47.9±4.9 | 1325±18 | 33.5±4.1 | 66.0±3.9 | 22.56±4.70 |
| **20** | 662±12 | 29.5±1.7 | 1506±23 | 15.8±1.4 | 70.8±2.2 | 28.65±5.21 |
| **27** | 696±12 | 27.5±2.0 | 1586±22 | 12.6±1.7 | 81.8±1.1 | 25.00±3.82 |
| **34** | 759±15 | 21.7±1.4 | 1738±29 | 9.5±1.2 | 94.9±2.3 | 10.10±2.91 |
|  |  |  | **EXCEL** |  |  |  |
| **13** | 650±10 | 48.7±4.3 | 1493±21 | 35.9±3.6 | 74.7±4.2 | 19.01±2.94 |
| **20** | 702±11 | 27.4±1.9 | 1617±21 | 14.0±1.6 | 81.8±2.3 | 26.25±4.31 |
| **27** | 753±14 | 25.5±1.6 | 1733±27 | 13.8±1.3 | 89.9±1.8 | 19.10±2.34 |
| **34** | 785±15 | 20.3±1.8 | 1804±30 | 9.8±1.5 | 93.3±2.0 | 10.36±2.07 |

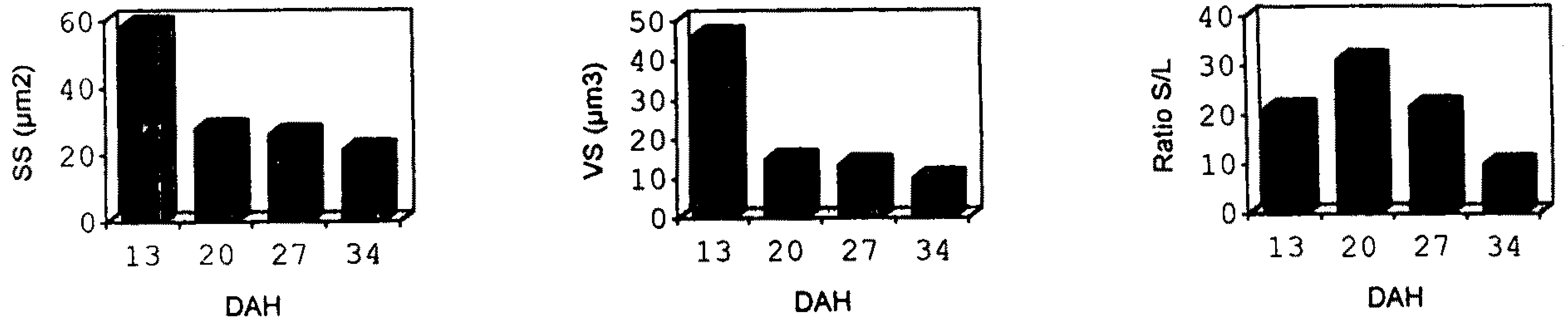
[1] PV, Proportion of L granules to total starch by volume



Surface area of L granules

Volume of L granules

Proportion of L to total starch by volume



Surface area of S granules

Volume of S granules

Ratio of No. S/L

FIGURE 1 - Changes in six starch granule traits four stages of kernel development pooled for three cultivars. Crookston, Mn, 1992.

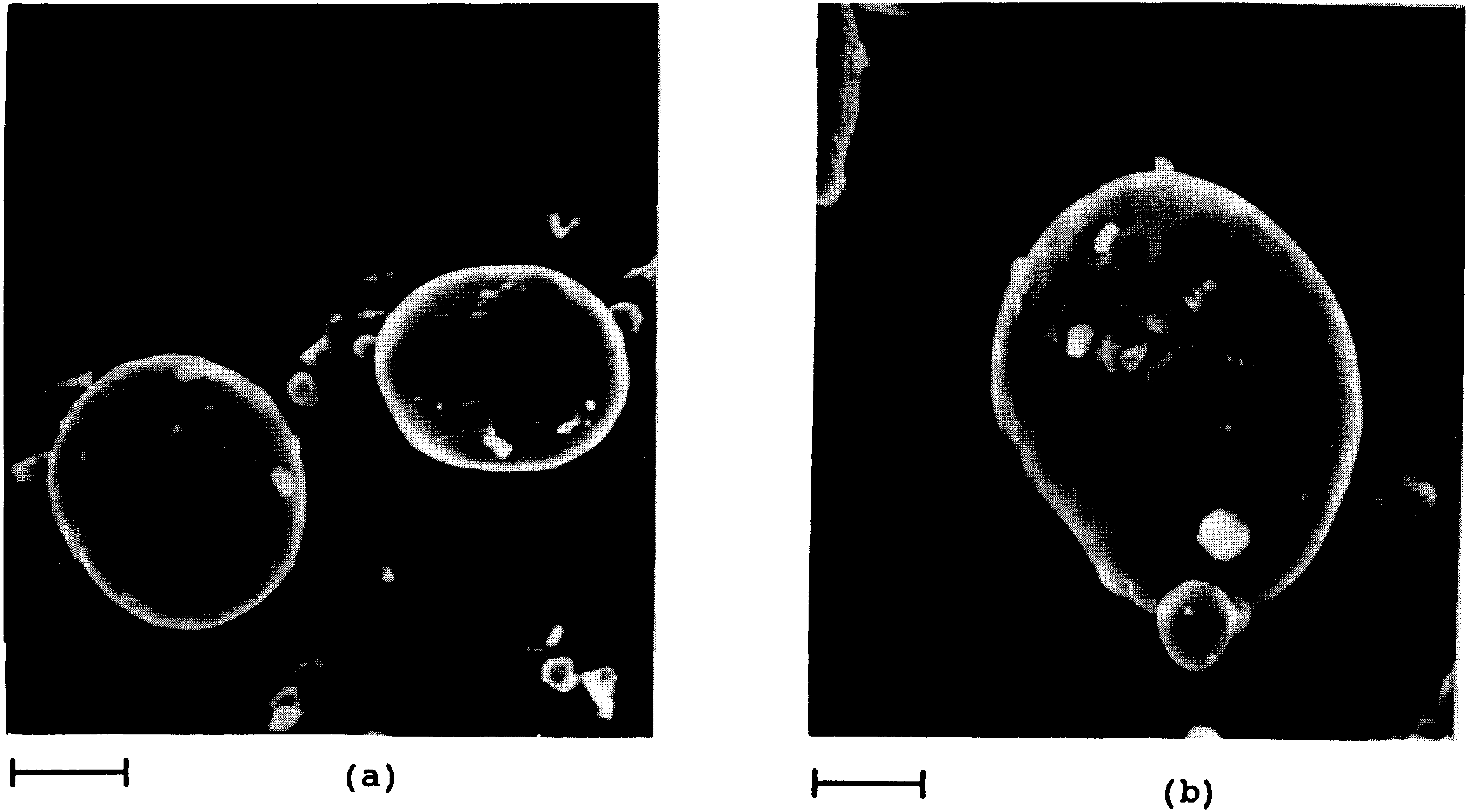


FIGURE 2 - Scanning electron micrographs of starch granules at 13 (a) and 34 (b) days after heading from cultivar Alexis. Bar marker represents 5 µm.

small granules are expected to reduce average small granule surface area and volume, while continuing growth of established granules would be an off-setting factor.

Ratio of number of small to large granules increased to a maximum of 31.0 at 20 DAH, then decreased to 9.9 at 34 DAH (Table 2). Apparently, the number of small granules produced at 20 DAH was smaller than the number of granules shifting from the small granule class into the large granule class, to result in the observed decrease of ratio of small to large granules as kernels matured.

Large granules of Alexis at two stages of development can be inspected in the scanning electron micrographs. Figure 2a shows two "large granules" at 13 DAH and Figure. 2b shows a more mature and larger "large granule" at 34 DAH from the same cultivar. Measurements made using this method agreed with those reported elsewhere (*12*).

## 4. CONCLUSIONS

In conclusion, it is clear that sizable changes in starch granule traits occurred during kernel development and that evaluation of genetic differences in starch granule traits must take stage of development into account. Starch granule evaluation should probably be delayed until near maturity.

## 5. SUMMARY

Starch, which constitute 60-64% of barley grains, occurs in the form of granules. These granules increase in size during kernel development and form two subpopulations. The objectives of this study were to evaluate the effect of kernel development stage on starch granule size. The effect of kernel development stage on starch granule traits was studied in three cultivars. A split-plot in time design with three replications was used. Kernels were sampled 13, 20, 27, and 34 days after heading (DAH). Granule traits varied substantially during kernel development. Surface area and volume of small granules decreased during kernel development. The proportion of large granules to total starch by volume also increased throughout kernel development, reaching 94.1% at 34 DAH. At 20 DAH, the ratio of number of small to large granules was at its maximum (31.0) and dropped to a minimum of 9.9 at 34 DAH. Means for surface area and volume of small granules appeared to decrease during kernel development due to shifting of larger "small granules" into the "large granule" class. This study demonstrates the need to account for kernel stage of development when evaluating starch granule sizes.

## 6. RESUMO

### (DESENVOLVIMENTO DOS GRÂNULOS DE AMIDO DA CEVADA)

O amido, constituindo aproximadamente 60 a 64% do peso dos grãos de cevada, ocorre em forma de grânulos que aumentam de tamanho durante o desenvolvimento dos grãos da cevada e formam duas subpopulações, denominadas grânulos "grandes" e "pequenos". Os objetivos deste estudo foram avaliar os efeitos do estádio de desenvolvimento do grãos de cevada na morfologia dos seus grânulos de amido. Três cultivares foram estudados em um delineamento em blocos casualizados com parcelas subdivididas e três repetições. Amostras de grãos foram coletadas aos 13, 20 27 e 34 dias após o florescimento (DAH). A morfologia dos grânulos de amido variou substancialmente durante o desenvolvimento dos grãos da cevada. Área superficial e volume dos grânulos pequenos diminuíram com o avanço do estádio de desenvolvimento. A proporção de grânulos grandes em relação ao volume total de amido também cresceu durante o desenvolvimento dos grãos, atingindo 94,1% aos 34 DAH. Aos 20 DAH, a proporção do número de grânulos pequenos para grânulos grandes atingiu seu máximo (31,0), tendo posteriormente reduzido para 9,9 aos 34 DAH. Médias de área superficial e volume dos grânulos pequenos aparentemente decresceram durante o desenvolvimento dos grãos devido à mudança de grânulos da classe "pequena" para a "grande". Este estudo demonstra a necessidade de se considerar o estádio de desenvolvimento dos grãos da cevada durante a avaliação da morfologia dos seus grânulos de amido.

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