BROWNING SENSITIVITY OF BUTTON MUSHROOMS

AMRAH WEIJN[1,2], M.M.M. TOMASSEN[1], S. BASTIAAN-NET[1], E.A.H.J. HENDRIX[3], J.J.P. BAARS[3], A.S.M. SONNENBERG[3], H.J. WICHERS[1,2], J. MES[1]
[1]Food & Biobased Research, Wageningen University and Research Centre,
Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands;
[2]Laboratory of Food Chemistry, Wageningen University and Research Centre,
Bomenweg 2, 6703 HD Wageningen, The Netherlands;
[3]Plant Research International – Mushrooms, Wageningen University and Research Centre, Droevendaalsesteeg 1,
6708 PB Wageningen,
The Netherlands
Amrah.Weijn@wur.nl , Jurriaan.Mes@wur.nl

ABSTRACT

To study the sensitivity of *Agaricus bisporus* mushrooms to bruising, a reproducible method was developed to apply mechanical damage to mushroom caps and quantify the subsequent discoloration. The newly developed bruising device can apply damage to the cap tissue of intact button mushrooms by a slip-shear sliding process in a fast and reproducible way. A protocol has been developed to obtain the most reliable and reproducible method to compare bruising sensitivity of different *A. bisporus* strains. The severity of the bruise is quantified with a computer image analysis system. Pictures of the bruised mushroom caps were taken under controlled lighting conditions and calibrated to a local reference. Image analysis software was developed to calculate the whiteness index (L-(3xb*), as defined by Hunter). This method of bruising and image based quantification was subsequently applied to a collection of wild, commercial and hybrid *A. bisporus* strains. A significant difference was found between bruising sensitive mushrooms and bruising tolerant mushrooms. A correlation was found between discoloration by the bruising device and discoloration caused by transportation of mushrooms on a conveyor belt. Less correlation was found between post-harvest discoloration of undamaged stored mushrooms and the bruising device. This indicates that discoloration caused by bruising or by storage of intact mushrooms might have different mechanisms.

Keywords: Bruising sensitivity; Computer image analysis; Button mushroom, *Agaricus bisporus*

INTRODUCTION

The quality of *Agaricus bisporus* button mushrooms is determined by colour, texture, cleanliness, maturity, flush number, and flavour. Of these, colour is the most important parameter because it is first perceived by consumers and discoloration decreases the commercial value [1]. Due to picking, handling and storage, discoloration reactions are initiated. Contact-based discoloration, or bruising, is caused by a mechanical process known as ‘slip-shear’ [1], a downwards force and a sideways movement, which can occur during picking by hand or by robotic picking equipment. Mechanical harvesting is more cost-efficient than picking by hand, but cannot be applied yet to serve the fresh market, as commercial strains are too sensitive to bruising. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones [2]. These products undergo subsequent reactions leading to the formation of the dark pigment melanin.

This project aims to develop a high throughput tool to quantify bruising sensitivity of mushrooms. This can support the selection of bruising resistant strains, suitable for mechanical harvesting of mushrooms for the fresh market. In order to breed for browning-tolerant lines and to study the molecular and biochemical processes in depth it will be necessary to determine the bruise-related browning sensitivity in a reproducible way. Burton [1] has developed a device to apply a slip-shear stress on mushroom slices. However, the damage applied was much stronger than occurs in practise and the method was too laborious to apply to a large number of samples.

Here we describe the use of a newly developed bruising device and image analysis system to quantify bruising sensitivity [3]. In order to develop a reliable and reproducible method, several parameters were studied in previous experiments. The parameters investigated were the influence of flush, the effects of the developmental stage of the mushrooms, the time between harvest and applying the bruise, and the time between bruising and analysing discoloration. A collection of *A. bisporus* strains was screened for their bruising sensitivity in order to analyse the phenotypic variation among strains. This method identified the genetic variation of bruising sensitivity among strains and now can support unravelling the molecular and biochemical basis for this trait. In this paper, we describe the correlation between discoloration by the developed bruising method and discoloration caused by a conveyor belt and discoloration after 7 days storage at 4 °C or 8 °C at 90% RH of the same mushroom varieties.

MATERIAL AND METHODS

Mushroom strains. Based on a previous collection screen of *A. bisporus* strains, several strains were chosen and cultivated. Eleven white mushroom strains were grown in seven replicates and four brown strains in two replicates. In each experiment, the strains were randomly distributed over the growing room. Strains used in this research originated from the Department of Plant Breeding at Wageningen UR and represent old and present day cultivars and wild collected varieties (ARP culture collection; [4]).

Mushroom growth. Spawn was prepared by boiling sorghum grain (*Sorghum bicolor*) for 20 min in water. After draining of water, gypsum (2.4% w/w) and chalk (0.7% w/w) were added before sterilizing. After sterilizing and cooling, grains were transferred to "full-gas microboxes" (Combiness, Gent, Belgium) and inoculated with a pure culture of an *A. bisporus* strain grown on agar (1% w/w malt extract, 0.5% w/w mycological peptone, 5 mM MOPS, pH 7). The colonization was completed in approximately two weeks with occasional shaking of the boxes to distribute colonized grains. Cultivation was performed in boxes (56 x 36 x 20 cm) filled with 16 kg of phase II compost [5]. Each box was inoculated with 110 ml of spawn. After a spawn run period of 14 days (air temperature at 21–23°C; RH 95%; 3500 ppm CO_2), casing soil was applied. After colonisation of the casing soil for 10 days at 21-23 °C, the casing layer was ruffled. Three days after ruffling, the boxes were vented at a rate of 0.075 °C/h towards 18 °C air temperature. At the same time, CO_2 was lowered at 35 ppm/h to a value of 1000 ppm and the RH was set at 90-92%. Depending on the strain, pins appeared between 3 and 10 days after the onset of venting. Harvest of the fastest growing strains started 7 days after venting and the majority of the strains produced 12-13 days after venting.

Bruising device. A new mushroom bruising device was constructed, which is able to apply damage to the cap tissue by a slip-shear sliding process using a spatula on a moving wheel (Figure 1). Ten mushrooms were fixed on a tray and bruised in series. The force with which the spatula presses on the mushroom caps can be adjusted by the weights placed on the horizontal bar on top of the machine. After initial experiments, the total weight of the spatula was set to 40

g. Mushrooms were kept at 20 °C constantly after harvest. After a recorded period of time, the mushrooms were placed into an illuminated cupboard (four Philips Fluotone TLD on each side of the tray) equipped with a photo-camera (JVC KY-F30E colour video camera with a JVC TV UM lens) and pictures were taken.

Computer image analysis. The developed quantitative image analysis system can measure various parameters from colour images of mushrooms and gives a quantitative value of discoloration. All images of a single experiment were calibrated using the standard Gretag-Macbeth colour checker (MSCCC) in order to compensate for small, unintentional variations in colour and illumination during the course of an experiment. A sheet of white paper was used to calibrate for the brightness and to compensate for variations in illumination within the cupboard. The brown discoloration was analysed using specially developed software (based on [6] and [7]) according to the CIE L*a*b* colour system [8].

Pictures taken give RGB colour space values which first need to be transformed to CIE XYZ values, for which the following transformation matrix is used [9],

0.49	0.31	0.20
0.17697	0.81240	0.01063
0.00	0.01	0.99.

The CIE XYZ values can then be transformed to CIE La*b* values [10], with the following formulae,

$L^* = 116 \int (Y/Y_n) - 16$

$a^* = 500 [\int (X/X_n) - \int (Y/Y_n)]$

$b^* = 200 [\int (Y/Y_n) - \int (Z/Z_n)]$.

Parameters were calculated for each mushroom individually and performed in the L, a*, b* colour space [8]. The software is not calibrated to the standard L, a*, b* colour space and consequently the outcome cannot be compared to results of other instruments. Images can only be compared mutually.

The position of each of the 10 mushrooms on the tray is identified and numbered automatically. The measurement area (Figure 2) is established such that 50% of the cap area is selected to exclude shadow effects. The bruising parameters used for the bruising applied by the bruising device are the whiteness index (WI) and the whiteness index difference (WI difference). WI is calculated as L-(3xb*), as defined by Hunter [11]. The WI difference is the difference between the average WI of a representative spot on the bruised area and the average WI of the control, not bruised tissue on the same mushroom (Figure 2). Tissue of mushrooms after the conveyer belt or after storage does not contain a control area. In this case the WI difference of the conveyor belt was calculated as the difference of the WI of the measurement area before and after the conveyer belt bruising at specific time points. The WI difference after storage was calculated as the difference between the WI of the measurement area before storage and the WI after 7 days of storage.

Outliert test. After calculating the bruising parameters from the normalised pictures, the data were analysed with Matlab. This program calculated the most deviant values (outliers) for each 10 mushrooms on a tray. This calculation is based on an analysis of variances of the trays. Mushrooms within a tray with a standardized residual of more than 2.3 were rejected and not used in further analysis.

Statistical analysis. Statistical analysis was performed using Genstat (13th edition). The linear mixed model (REML) was used to calculate significant differences between strains with a level of significance of 0.05. This was done with the least significant difference (LSD).

RESULTS

Developed bruising method. In previous experiments, the optimal method to bruise mushroom with the bruising device was determined [3]. Medium sized (35-55mm) closed or veiled mushrooms from the first and second flush were used to analyse bruise-related browning sensitivity by applying three times a slip shear force with the spatula over the cap tissue (with the weight of the spatula adjusted to 40 g). Mushrooms were bruised within 4 h after harvest. Pictures of the bruised mushrooms are taken 60 min after bruising and analysed with the computer image analysis system. The computer image analysis system can be used to determine the WI of a specific bruised area on the cap of the mushroom or of the whole cap surface, which is set at 50% of the total cap area (circle in Figure 2).

Figure 1: Bruising device. The spatula can be moved by hand over the tray. The weights in the horizontal bar can be changed (set at 40 g in our experiments).

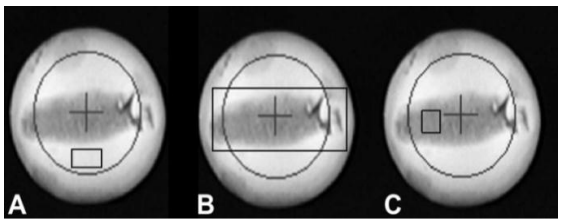

Figure 2: Computer image analysis areas. A control area. B bruise boundary. C bruised area. The circle on each mushroom is the measurement area (set at 50% of the surface).

Bruise-related browning sensitivity of 15 different *A. bisporus* strains. The method described above was used to characterize the genetic variation of bruising sensitivity among a small collection of 11 white and four brown *A. bisporus* strains (Figures 3 and 4). Comparison of the bruising sensitivity of the tested strains indicated that a considerable variation in bruising sensitivity exists among button mushrooms. At this stage of comparing bruising sensitivity of white and brown capped mushrooms, we are not sure whether WI differences can be influenced by large differences in cap background colours. That is why both types of cap coloured mushrooms were analysed separately (Figure 3 for white capped strains and Figure 4 for brown capped strains). Among the white strains, it was found that some of the commercial strains, such as commercial hybrids 1, 2 and 3, were among the least sensitive strains and wild-type white strain 1 also showed low bruising sensitivity (Figure 3). Among the white button mushroom strains, very sensitive strains could also be identified, such as wild-type white strain 7. For the brown cap coloured strains, only wild strains were tested and these showed a significant difference in bruising sensitivity. Within this small selection, it was found that the two tolerant brown strains have a lower WI difference than found for the most tolerant white strain. Several strains were included in more than one experiment and it was shown that the classification of discoloration after bruising into tolerant, moderate and sensitive was reproducible (data not shown).

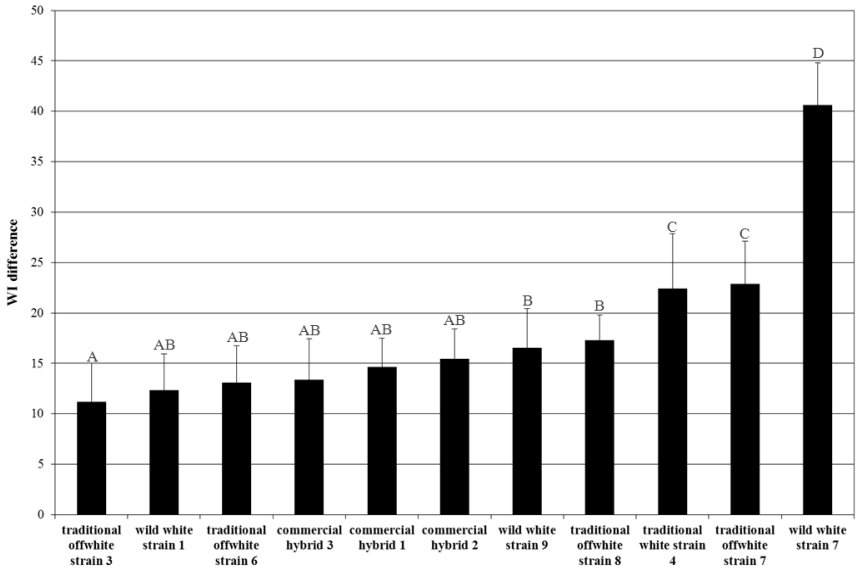

Figure 3: WI difference of 11 white button mushrooms from flush 1, 60 min after bruising. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level.

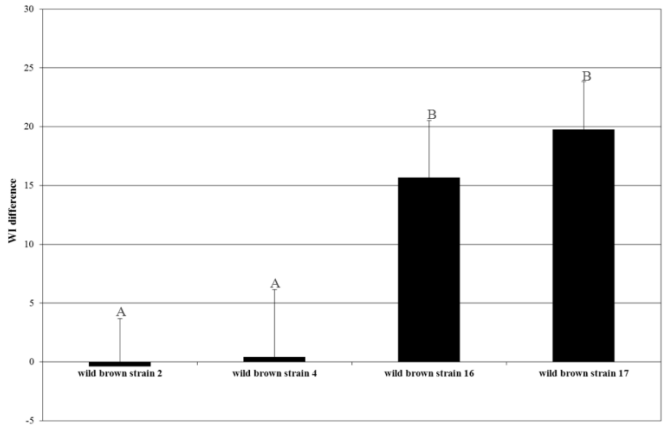

Figure 4: WI difference of 4 brown button mushrooms of flush 1, 60min after bruising. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level.

Bruising button mushrooms with a conveyor belt. Mushrooms of seven strains were bruised with a conveyor belt to compare the discoloration after bruising with the results of the in-house developed bruising device. A mushroom grower was visited to check the times of bruising during mechanical harvesting and sorting. Approximately 20 falling motions occurred during the sorting process. Mushrooms were applied 5 times in a row on a conveyor belt 5 m in total length (Figure 5). Mushrooms from the second flush were used, and at least 10 mushrooms per time point were tested. The WI of the whole cap was measured and not of a specific bruised part. This was done by using the measurement area (the circle in Figure 2). A comparison was made between control (not bruised) mushrooms at T = 0 min and bruised mushrooms at three time points, 5, 60, and 120 min after bruising.

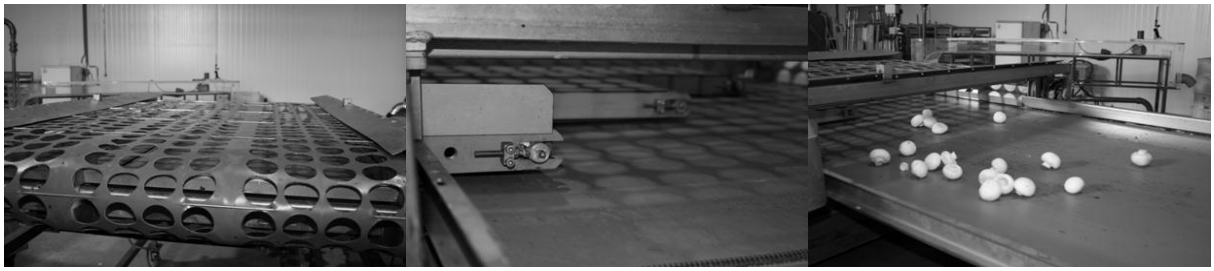

Figure 5: Conveyor belt used for the bruising experiment (from WeBe Engineering)

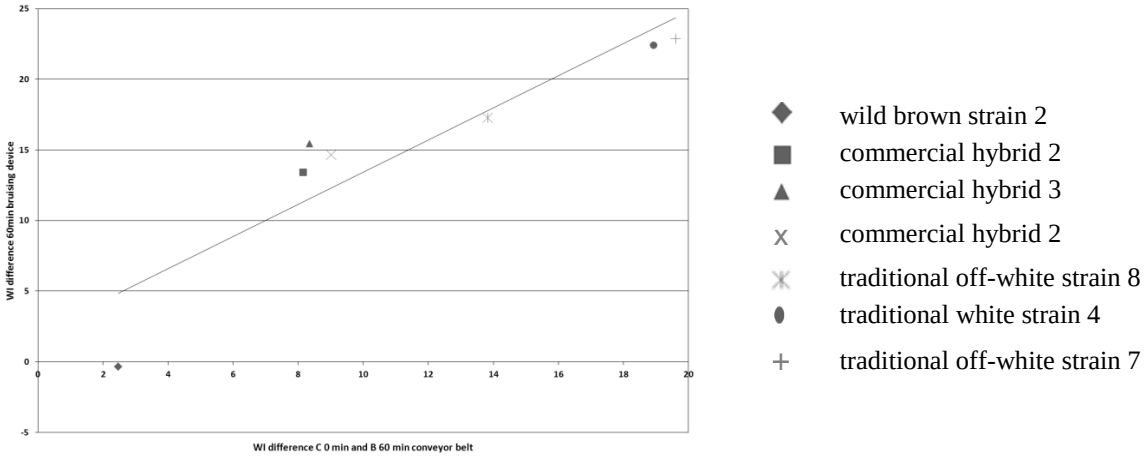

Figure 6: Correlation between conveyor belt bruising and discoloration after bruising with the bruising device. Y = 1.1388x + 2.0361, R^2 = 0.8444 (brown strain left out, gives Y = 0.749x + 7.9264, R^2 = 0.9612). C 0 min = control at T = 0 min, B 60 min = bruised T = 60 min

In figure 6 the correlation is shown between the WI difference 60 min after bruising with the bruising device and the WI difference of the conveyor belt. The WI difference of the conveyor belt is calculated as the difference between WI control 0 min (C 0 min) and WI bruised 60 min (B 60 min) after bruising with the conveyor belt. A correlation of 0.8444 could be found for the 7 strains used. The in-house developed bruising device gives a bruising discoloration comparable to industrially-used machines. When the only brown strain used was left out, the correlation is higher (R^2 = 0.9612).

Shelf Life Performance. Flush 2 mushrooms of 11 different strains were stored for 7 days at 4 ^{O}C or 8 ^{O}C at 90% humidity in boxes with a lid with small holes (as used in Dutch supermarkets). Pictures of the mushrooms were taken at the starting time point and at day 7. Mushrooms were not bruised, so discoloration upon storage was analysed. The WI of the measurement area of the mushroom was analysed (Figure 2). The mushrooms were weighed at day 0 and day 7 to follow the change in weight upon storage, to achieve approximately the same weight in each box and that boxes were filled completely. In general both white and brown button mushrooms discolour more at 8 ^{O}C than at 4 ^{O}C, except for wild white strain 1 and traditional off-white strain 3 (Figure 7 for white capped strains and Figure 8 for brown capped strains). The difference between the discoloration upon storage at 4 ^{O}C and 8 ^{O}C is not the same for every strain. As shown before, the commercial hybrids 1, 2 and 3 show the least discoloration. For the other white and brown strains that were analysed, there is less correlation between the discoloration after bruising compared to the discoloration after storage. This can be due to the fact that mushrooms strains showed a difference in development during this storage period; some strains were open after 7 days and sporulated, while some strains still contained closed or veiled mushrooms. Smith et al. [12] followed mushroom development (cap opening) during

storage at 18 ^{O}C for *A. bisporus* U3 and 2 *Agaricus bitorquis* strains and found a difference in the rate of maturation. One of the *A. bitorquis* strains developed much slower and, even after 5 days of storage, stage 3 mushrooms were found in most cases (based on Hammond and Nichols; [13]). The variation in discoloration after storage is less in the brown mushroom strains than found with the bruising device. The weight loss after 7 days storage was always higher at 8 ^{O}C than 4 ^{O}C, except for traditional off-white strain 3 (Table 1).

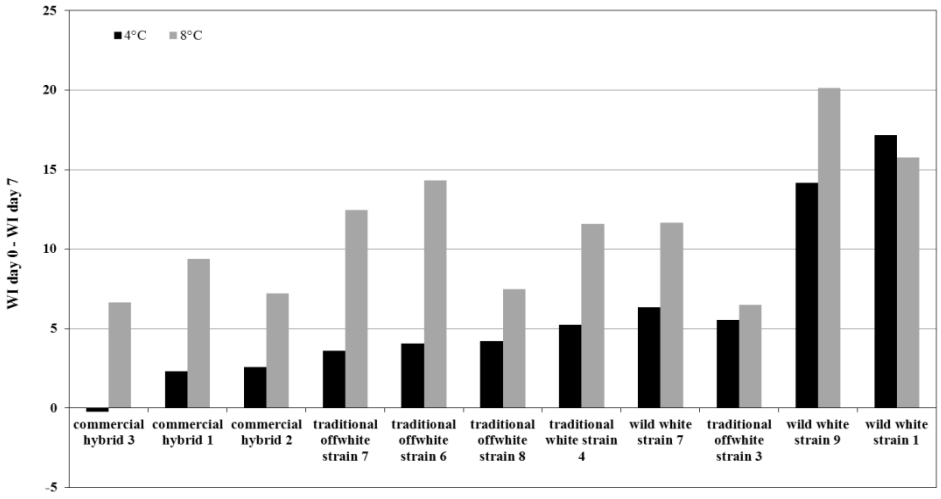

Figure 7: Difference between WI day 0 and WI day 7 after storage at 4 **^{O}C** or 8 **^{O}C** of 11 white button mushroom strains.

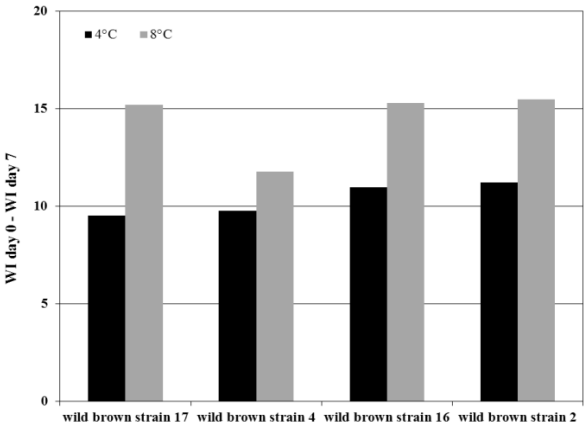

Figure 8: Difference between WI day 0 and WI day 7 after storage at 4 **^{O}C** or 8 **^{O}C** of four brown button mushroom strains.

Table 1: Weight loss in g per mushroom after storage for 7 days.

Strain	at 4 ^{O}C	at 8 ^{O}C
wild white strain 1	0.42	0.85
wild white strain 9	0.33	1.05
commercial hybrid 2	0.54	1.60
commercial hybrid 1	0.50	1.58
commercial hybrid 3	0.36	2.09
traditional off-white strain 3	1.93	1.08
wild white strain 7	0.35	1.11
traditional white strain 4	0.18	1.86
traditional off-white strain 6	0.44	0.79
traditional off-white strain 7	0.31	1.08
traditional off-white strain 8	0.28	0.96
wild brown strain 4	0.05	1.51
wild brown strain 2	0.36	0.69
wild brown strain 17	0.59	1.92
wild brown strain 16	0.33	2.53

DISCUSSION

Bruising-related discoloration of mushrooms is an important loss of quality caused by picking, conveyor belts or storage. In order to unravel the mechanisms behind bruising sensitivity, to compare pre- or post-harvest treatments and to breed for bruising tolerant strains, it will be necessary to have access to a reliable bruising quantification method. In previous experiments,

factors were studied that influence the reproducibility, internal sample variation, and quantification of the bruise-related brown discoloration. This resulted in a standard protocol to determine bruising sensitivity in a quantitative manner [3]. Quantification of the discoloration is based on measuring the WI both of bruised and undamaged tissue of the same cap and subtracting the WI value of the undamaged tissue from the WI value of the bruised tissue.

Here, the bruising method is compared with bruising by a conveyor belt and by discoloration during cold storage using a collection of strains with different degrees of bruising sensitivity. Although the selection was chosen randomly, it can be stated that there was a larger variation in sensitivity within the brown strains than in the white strains upon bruising with the bruising device.

The results indicate that there is a correlation between the bruising device induced discoloration and conveyer belt damaging sensitivity. Including more strains and mushrooms from the same flush in this type of analysis will allow to further detail the correlation between different mushroom strains. Less correlation was found between the discoloration after cold storage of undamaged mushrooms and 60 minutes after bruising with the device. Possibly, different mechanisms are involved in causing storage or bruising-related discoloration, as the stored mushrooms were not bruised. In addition, the loss of water, membrane damage and enzyme activation can cause a different response and result in higher or lower browning sensitivity. The difference in shelf life performance of different strains is also an important factor. For some strains, all mushrooms opened during 7 days of storage and for other strains the mushrooms were closed or veiled. Although not correlating to bruising sensitivity, the possible genetic variation in shelf-life performance and discoloration of the strains tested can be of interest to the industry for increasing shelf life performance by breeding.

The bruise-related browning sensitivity quantification method can be used for in- depth research to unravel the molecular and biochemical pathways behind the trait. These studies can be focused on substrate availability and enzyme activity, as reviewed by Jolivet et al. [2]. Different starting substrates lead to the formation of different types of melanin, of which the intermediate products have different colours. Dedicated transcriptomics, metabolomics and proteomics can be used to unravel the molecular and biochemical pathways behind brown discoloration.

ACKNOWLEDGEMENTS

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