**Nutrient depletion modifies cell wall adsorption activity of wine yeast**

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**Abstract** Yeast cell wall is a structure that helps yeasts to manage and respond to many environmental stresses. The mannosylphosphorylation is a modification in response to stress that provides the cell wall with negative charges able to bind compounds present in the environment. Phenotypes related to the cell wall modification such as the filamentous growth in *Saccharomyces cerevisiae* are affected by nutrient depletion. The present work aimed at describing the effect of carbon and/or nitrogen limitation on the aptitude of *S. cerevisiae* strains to bind coloured polyphenols. Carbon- and nitrogen- rich or deficient media supplemented with grape polyphenols were used to simulate different grape juice conditions - early, mid, 'adjusted' for nitrogen, and late fermentations. In early fermentation condition, the R+G+B values range from 106 (high adsorption, strain Sc1128) to 192 (low adsorption, strain Σ1278b), in mid-fermentation the values range from 111 (high adsorption, strain Sc1321) to 258 (low adsorption, strain Sc2306), in 'adjusted' for nitrogen conditions the values range from 105 (high adsorption, strain Sc1321) to 194 (low adsorption, strain Sc2306) while in late fermentation conditions the values range from 101 (high adsorption, strain Sc384) to 293 (low adsorption, strain Sc2306). The effect of nutrient availability is not univocal for all the strains and the different media tested modified the strains behaviour. In all the media the strains show significant differences. Results demonstrate that wine yeasts decrease/increase their parietal adsorption activity according to the nutrient availability. The wide range of strain variability observed could be useful in selecting wine starters.

**Keywords** carbon and nitrogen availability· grape polyphenols· nutrient depletion ·parietal adsorption activity·wine *Saccharomyces cerevisiae*

## Introduction

Yeast cell wall is a highly dynamic structure that helps yeasts to manage and respond to many environmental stresses (García-Rodríguez et al. 2005; Klis et al. 2006). Its composition is not only controlled by cell-cycle but also depends on environmental conditions such as nutrient availability (Klis 1994). In response to this, cell can make considerable adjustments to the composition and structure (Kapteynet al. 1999) by defined programs of gene expression (Siderius and Mager 2003). In *Saccharomyces cerevisiae*, proteins encoded by FLO genes, known as flocculins, confer adherence to agar, solid surfaces and other yeast cells (Guo et al. 2000; Reynolds and Fink 2001; Douglas et al. 2007) resulting in formation of biofilm, mat colonies, and grow as filamentous cells. Those phenotypes are found related to glucose and carbon availability (Gimeno et al. 1992; Cullen and Sprague 2000; Reynolds and Fink 2001; Gagiano et al. 2002; Kuchin et al. 2002; Reynolds et al. 2008) and controlled by different signalling nutrient-sensing pathways (Cullen and Sprague 2012; Karunanithi et al. 2012). Recently, Sidari et al. (2014) reported the behavioural variability of wild strains of *S. cerevisiae* toward biofilm-like phenotypes when grown on media with nutrient limitation. Also, they demonstrated that polyphenols present in grapes and must affect these phenotypes in strain dependent way.

The mannosylphosphorylation of yeast cell wall is a major modification also in response to stress (Kulaev and Kulakovskaya, 2000) that provides the cell wall with negative charges. It determines variation in the strain adsorption of cationic dyes. In *S. cerevisiae* the phosphorylation of N-oligosaccharides is a process involving gene products. Among these:MNN6 gene encodes a mannosylphosphate transferase and MNN4 gene is a positive regulator; in fact, the amount of Mnn4p has been reported to be a limiting factor for mannosylphosphorylation (Odaniet al. 1997; Jigami and Odani, 1999); the KTR and MNN1 mannosyltransferase gene families (Lussier et al. 1999); MNN2 gene (Olivero et al. 2000); different LDB genes (Mañas et al. 1997; Corbacho et al. 2004); different nonessential genes of diverse functional categories (Corbacho et al. 2005).

Nutrient stress should lead to an increase in negative charges of the cell wall resulting in an increase in the yeast adsorption activity of compounds present in the surrounding environment.

During winemaking, yeast can adsorb grape polyphenols on the negative charge of its cell wall (Morata et al. 2003; Medina et al. 2005; Caridi 2006). This cell wall adsorption activity plays a role, together with other factors, in the final chromatic properties of wines. Therefore, strains with high adsorption activity lead to an impoverishment of

colour matter in the wine and vice versa for strains with low adsorption activity. In addition, during fermentation other factors affect the colour of wine such as the yeast release of pyruvic acid and acetaldehyde that form stable derivatives reacting with anthocyanins (Medina et al. 2005). Considering the ever-growing quest for yeasts with optimized or novel oenological properties (Pretorius 2000), the yeast colour adsorption activity is one of the factors to consider in wine strain selection.

To quickly screen wine *S. cerevisiae* strains for their adsorption aptitude, the Grape Skin agar (GraSki), rich both in carbon and nitrogen, has been proposed (Caridi et al. 2007). Yeasts growing with white biomass in GraSki tend to not adsorb coloured polyphenols while yeasts growing with dark brown biomass have opposite aptitude. The former strains are useful to produce red wines while the latter ones to produce white wines. Recently, Caridi et al. (2015) have reported the combined use of GraSki and microvinification trials in presence or in absence of $SO_2$ to screen a great number of *S. cerevisiae* strains for their colour adsorption behaviour in the defined medium -both as singular colour parameter red, green, and blue and as sum of them - and in grape must.

We were interested in studying if, analogously to yeast biofilm-like properties, the yeast ability to adsorb coloured polyphenols is affected by nutrient availability.

The present work aims at describing the effect of nutrient depletion on coloured polyphenolic adsorption activity of wine yeasts.

## Materials and methods

### Yeast strains

The study was carried out using thirteen *S. cerevisiae* strains: eleven strains isolated from Calabrian grapes and musts, previously identified by RFLP, and studied for their biofilm-like behaviour also in presence of polyphenols (Sidari et al. 2014) and two laboratory strains -Σ1278b, (MATa), which has adhesive and filamentous phenotypes (Reynolds and Fink 2001), and BY4742, (MATα, Δflo8), which has a non-adhesive phenotype (Euroscarf collection, Frankfurt).

### Culture conditions

The strains were grown in Yeast Peptone Dextrose broth (YPD - 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose), and on Yeast Peptone Dextrose agar (YPD - 10 g/l yeast extract, 20 g/l peptone, 20 g/l or 1 g/l glucose, 20 g/l agar). The defined media used were: GraSki agar (60 g/l dried black grape skins, 50 g/l citric acid monohydrate, 25 g/l disodium hydrogen phosphate, 20 g/l glucose, 7.5 g/l peptone from casein, 4.5 g/l yeast extract, and 20 g/l agar - pH 3.50) (Caridi 2013), modified GraSki for glucose (1 g/l, Sidari et al. 2014) and Synthetic Low Ammonium Dextrose (SLAD - 1.7 g/lDifco™ YNB without aminoacids, 0.006 g/l ammonium sulfate, 1.3 g/l essential aminoacids for nutritional auxotrophies (Treco and Lundblad, 1993), 20 g/l glucose) (Zaragoza and Gancedo 2000). Dried black grape skins used to prepare the GraSki agar were added to supplement SLAD with a mix of polyphenols(60 g/l dried black grape skins, 1.7 g/l Difco™ YNB without aminoacids, 0.006 g/l ammonium sulfate, 1.3 g/l essential aminoacids, 20 or 1 g/l glucose, and 20 g/l agar - pH 3.50). These media simulated nutritional condition of high carbon and nitrogen concentration (GraSki 20 g/l of glucose - early fermentation), rate limiting nitrogen and high concentration in carbon (SLAD 20 g/l of glucose - mid-fermentation), rate limiting carbon and high concentration in nitrogen (GraSki 1 g/l of glucose - 'adjusted' fermentation for nitrogen) (Sidariet al.2014), and rate limiting carbon and nitrogen (SLAD 1 g/l of glucose - late fermentation). The YPD agar was used as control medium.

Assay of coloured polyphenolic adsorption on yeast cell wall

The strains from liquid YPD cultures were grown in YPD agar at 28°C for 2 days, streaked on Petri plates containing GraSki, SLAD, and YPD media, and anaerobically incubated at 28°C for 10 days. Then, yeast biomass was carefully mixed, taken with a loop, photographed, and processed for colour components using Red-Green-Blue (RGB) analysis by Adobe Photoshop CS2. Each colour component was read in quadruplicate. The values range from 0 (black) to 255 (white). High R, G, and B values correspond to low adsorption activity (light biomass); low R, G, and B values correspond to high adsorption activity (dark biomass). To give a global idea of the yeast adsorption aptitude the sum of red, green, and blue values (R+G+B) was considered.

Statistical analysis

Data were statistically analysed by StatGraphics Centurion XVII for Windows XP from StatPoint according to the *Least Significant Differences of Fisher*, confidence level of 95%.

**Results**

The strain aptitude to adsorb coloured polyphenolic compounds was tested in conditions simulating different phases of fermentation. The strains were grouped into homogeneous groups; a homogeneous group defined as a group of means within which there are no statistically significant differences. Figure 1 shows means, standard deviations and homogeneous groups of the R parameter for all the strains tested in the four conditions simulating different phases of fermentation. The minimum value (high adsorption) was 55 for strain Sc1321 in GraSki with 1 g/l of glucose while the maximum (low adsorption) value was 140 for strain Sc2306 in SLAD with 1 g/l of glucose. For this parameter the strains were distributed in 9, 7, 9, and 9homogeneous groups (letters above the histogram bars of each medium read in horizontal lines) in GraSki20 g/l of glucose, GraSki with 1 g/l of glucose, SLAD 20 g/l of glucose, and SLAD with 1 g/l of glucose, respectively.

Figure 2 shows means, standard deviations and homogeneous groups of the G parameter for all the strains tested in the four conditions simulating different phases of fermentation. The minimum value was 23 for strains Sc632 and Sc1321 respectively in SLAD with 1 g/l of glucose and SLAD with 20 g/l of glucose while the maximum value was 66 for strain Σ1278b in SLAD with 1 g/l of glucose. For this parameter the strains were distributed in 8, 5, 8, and 9 homogeneous groups (letters above the histogram bars of each medium read in horizontal lines) in GraSki 20 g/l of glucose, GraSki with 1 g/l of glucose, SLAD 20 g/l of glucose, and SLAD with 1 g/l of glucose, respectively.

Figure 3 shows means, standard deviations and homogeneous groups of the B parameter for all the strains tested in the four conditions simulating different phases of fermentation. The minimum value was 21 for strain SLAD with 1 g/l of glucose while the maximum value was 67 for strain Sc2306 in SLAD with 1 g/l of glucose. For this parameter the strains were distributed in 6, 5, 4, and 9 homogeneous groups (letters above the histogram bars of each medium read in horizontal lines) in GraSki 20 g/l of glucose, GraSki with 1 g/l of glucose, SLAD 20 g/l of glucose, and SLAD with 1g/l of glucose, respectively.

Significant differences among the media were observed. In details, for the R parameter between: GraSki with 20 g/l of glucose and SLAD with 20 g/l of glucose, GraSki 20 g/l of glucose and SLAD 1 g/l of glucose, SLAD 20 g/l of

glucose and GraSki 1 g/l of glucose, SLAD 20 g/l of glucose and SLAD 1 g/l of glucose, and GraSki 1 g/l of glucose and SLAD 1 g/l of glucose. For the G parameter between: GraSki 20 g/l of glucose and SLAD 1 g/l of glucose, GraSki 1 g/l of glucose and SLAD 1 g/l of glucose while for the B parameter between: GraSki 20 g/l of glucose and SLAD 1 g/l of glucose.

For each biomass colour components, all the strains grown on YPD agar exhibited values higher (142-160 for red, 95-139 for green, 56-115 for blue) than those obtained on the other media (data not shown).

Table 1 reports the strain adsorption behaviour as R+G+B values comparing early fermentation conditions with mid-fermentation (GraSki 20 g/l vs. SLAD 20 g/l), 'adjusted' for nitrogen (GraSki 20 g/l vs. GraSki 1 g/l), and late fermentation conditions (GraSki 20 g/l vs. SLAD 1 g/l). For each strain and experimental condition the value of the R parameter (number in bracket) is also reported. The thirteen yeast strains showed significant differences in all the media tested and the effect of nutrient availability is not univocal for all the strains.

In early fermentation conditions, the R+G+B values range from 106 (high adsorption, strain Sc1128) to 192 (low adsorption, strain Σ1278b). The strains Sc384, Sc1128, and BY4742 adsorbed highly coloured compounds, the strains Sc2306 and Σ1278b had opposite aptitude while the other strains had intermediate aptitude. Compared to the early fermentation condition, in the mid-fermentation (rate limiting nitrogen),the R+G+B values range from 111 (high adsorption, strain Sc1321) to 258 (low adsorption, strain Sc2306). This condition determined an increase in the coloured compounds adsorbed (that result in an impoverishment of colour matter in the wine) in the strains Sc1240, Sc1321, Sc1340, and Sc1741. In the 'adjusted' for nitrogen conditions (rate limiting carbon), the R+G+B values range from 105 (high adsorption, strain Sc1321) to 194 (low adsorption, strain Sc2306). The strains Sc1240, Sc1321, Sc1340, Sc1741, Sc2621, and Σ1278b showed an increase in adsorption of coloured compounds. In the late fermentation conditions (rate limiting carbon and nitrogen), the R+G+B values range from 101 (high adsorption, strain Sc384) to 293 (low adsorption, strain Sc2306). An increase in the adsorption of coloured compound was observed in the strains Sc384, Sc632, Sc1240, and Sc1591. Decrease in the adsorption aptitude were observed in strains Sc1128, Sc1326, Sc2306, and BY4742 grown in media simulating the 'adjusted' for nitrogen conditions (rate limiting carbon), in the same strains plus the strains Sc384, Sc632, Sc2621, and Σ1278b grown in mid-fermentation conditions (rate limiting nitrogen), and in strains Sc1128, Sc1326, Sc1340, Sc1741, Sc2306, Sc2621, BY4742, and Σ1278b grown in late fermentation conditions (rate limiting carbon and nitrogen). Some strains showed an unchanged adsorption aptitude growing in the different nutrient availability.

198

199 **Discussion**

200 The strain behaviour towards the coloured polyphenolic compounds is attributable to a defined cell wall
201 composition in terms of mannoproteins and quantity of phosphate. The more is the phosphate content the more are
202 the compounds adsorbed, similarly to the alcian blue adsorption to the yeast cell wall (Odani et al. 1997).

203 Progress in the fermentation (media simulating mid, 'adjusted', and late fermentation) determines a modification
204 in the yeast aptitude with increase in pigment adsorption (30.77% of strains grown in condition of either nitrogen or
205 carbon and nitrogen limitation; 46.15 % of the strains grown in condition of carbon limitation). This modification in
206 the yeast behaviour could be explained by the fact that nutritional stress induces an increase in phosphate transfer to
207 the mannoproteins, mediated by one or more gene products and regulators (see Introduction section) among which
208 the mnn6p and mnn4p, resulting in more cell wall negative charges able to bind coloured compounds. Other strains
209 showed the opposite behaviour (61.54% of the strains grown in condition of either nitrogen or carbon and nitrogen
210 limitation; 30.77 % of the strains grown in condition of carbon limitation).This modification in the yeast behaviour
211 (that results in the maintenance of colour matter in the wine) could be explained by a lacking of phosphate transfer to
212 the mannoproteins. These strains could be either defective in mnn4p, which is a positive regulator of the mnn6p
213 (Odani et al. 1997), or have expressed some other genes involved in the phosphate transfer (see Introduction section).
214 Less frequent was the unchanged strain behaviour in the different media (7.70% and 23% of the strains grown either
215 in nitrogen or carbon limitation, respectively). Probably, for some strains the polyphenols allow them to overcome
216 the nutrient stress by activating or not the complex process involving several genes (see Introduction section).
217 Analogously to the Calabrian strains, the two laboratory strains show a not univocal colour adsorption behaviour
218 when grow in different nutrient availability. The strain BY4742 shows high adsorption in GraSki with 20 g/l of
219 glucose while it decreases the adsorption aptitude in all the other media. The strain Σ1278b shows low adsorption in
220 GraSki with 20 g/l of glucose, an increase in the adsorption aptitude in GraSki with 1 g/l of glucose while a decrease
221 in the two SLAD media.

222 The colour values obtained by Photoshop, analysing biomass of the strains grown in YPD, was expectable; in
223 fact, high value means white or light biomass and it is due to the lack of coloured polyphenolic compounds in the
224 medium formulation available for yeasts. This strain behaviour confirms that the YPD is not useful to screen yeast
225 strains for pigment adsorption activity. On the other hand, to screen this adsorption activity it is possible to use both

the GraSki and the SLAD supplemented with grape skins, with a pH of 3.50 to simulate the grape must environment and to allow the best colour expression of grape pigments.

Our results demonstrate for the first time that nutritional composition affects the yeast adsorption aptitude toward coloured polyphenols. Consequently, if grape must is deficient in carbon, nitrogen or both, the adsorption activity of wine yeasts may be modified in comparison to that one exhibited in optimal presence of nutrients, so the chromatic properties of the wine could be different from those expected. Moreover, the nutrients affect the yeast aptitude to adsorb pigments in a strain dependent way. In conclusion, during the selection process of starter cultures for winemaking it seems interesting to consider the strain aptitude to adsorb/not adsorb grape pigments taking also into account its possible modification due to nutrient stress.

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**Figure legends**

**Fig. 1**Means and standard deviations (error bars of four replicates) of R parameter for the thirteen strains tested in media simulating early fermentation conditions (GraSki 20 g/l of glucose), mid-fermentation conditions (SLAD 20 g/l of glucose plus grape skins as source of polyphenols, 'adjusted' fermentation with nitrogen source (GraSki 1 g/l of glucose), and late fermentation conditions (SLAD 1 g/l of glucose plus grape skins as source of polyphenols). For each media letters above the histogram bars read in horizontal lines indicate the strains distribution in homogeneous groups

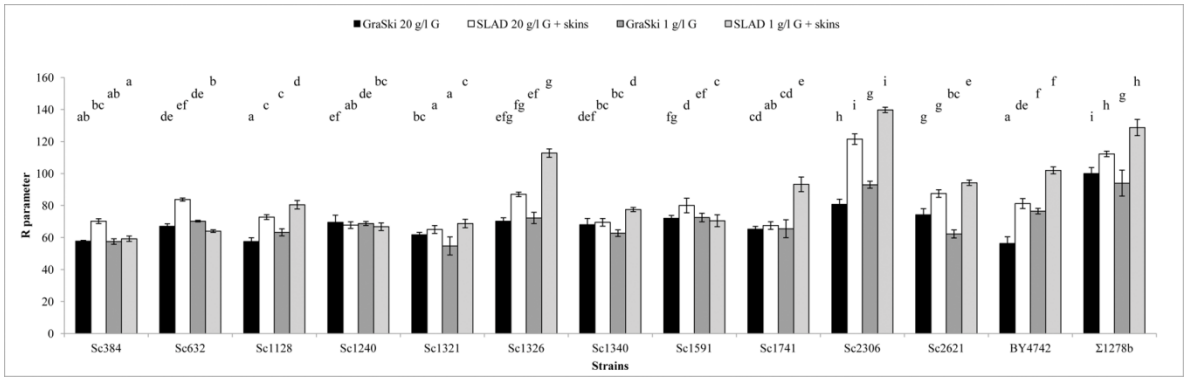
**Fig. 2** Means and standard deviations (error bars of four replicates) of G parameter for the thirteen strains tested in media simulating early fermentation conditions (GraSki 20 g/l of glucose), mid-fermentation conditions (SLAD 20 g/l of glucose plus grape skins as source of polyphenols, 'adjusted' fermentation with nitrogen source (GraSki 1 g/l of glucose), and late fermentation conditions (SLAD 1 g/l of glucose plus grape skins as source of polyphenols). For each media letters above the histogram bars read in horizontal lines indicate the strains distribution in homogeneous groups

**Fig. 3** Means and standard deviations (error bars of four replicates) of B parameter for the thirteen strains tested in media simulating early fermentation conditions (GraSki 20 g/l of glucose), mid-fermentation conditions (SLAD 20 g/l of glucose plus grape skins as source of polyphenols, 'adjusted' fermentation with nitrogen source (GraSki 1 g/l of glucose), and late fermentation conditions (SLAD 1 g/l of glucose plus grape skins as source of polyphenols). For each media letters above the histogram bars read in horizontal lines indicate the strains distribution in homogeneous groups

**Table 1** Quantification of coloured polyphenols adsorption aptitude, as R+G+B, of the thirteen strains of *Saccharomyces cerevisiae* grown in media simulating early fermentation conditions (GraSki 20 g/l of glucose), mid-fermentation conditions (SLAD 20 g/l of glucose plus grape skin as source of polyphenols), 'adjusted' fermentation with nitrogen source (GraSki 1 g/l of glucose), and late fermentation conditions (SLAD 1 g/l of glucose plus grape skin as source of polyphenols). Data represent means of four replicates. Superscript letters are the homogeneous groups (*Least Significant Differences of Fisher*, confidence level of 95 %). The arrows pointing up or down and the equal sign indicate the modification of the strains adsorption aptitude compared to the GraSki 20 g/l of glucose. The numbers in brackets define the values of the R parameter

| | **Media** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Strains** | **High C High N** | **High C Rate limiting N** | | **Rate limiting C High N** | | **Rate limiting C Rate limiting N** | |
| | **GraSki 20 g/l G[a]** | **SLAD 20 g/l G** | | **GraSki 1 g/l G** | | **SLAD 1 g/l G** | |
| Sc384 | 108±1.7$^{a}$ (58) | 136±7.8$^{c}$ (70) | ↓ | 107±4.1$^{ab}$ (58) | = | 101±2.1$^{a}$ (59) | ↑ |
| Sc632 | 122±1.0$^{bcd}$(67) | 141±1.4$^{cd}$ (84) | ↓ | 124±2.9$^{cd}$ (70) | = | 109±1.7$^{b}$ (64) | ↑ |
| Sc1128 | 106±2.4$^{a}$ (58) | 122±2.2$^{b}$ (73) | ↓ | 118±4.3$^{bc}$ (63) | ↓ | 133±4.1$^{e}$ (81) | ↓ |
| Sc1240 | 128±7.0$^{def}$ (70) | 120±10.4$^{ab}$ (68) | ↑ | 123±3.8$^{cd}$ (69) | ↑ | 118±3.5$^{c}$ (67) | ↑ |
| Sc1321 | 115±4.5$^{ab}$ (62) | 111±7.9$^{a}$ (65) | ↑ | 105±10.2$^{a}$ (55) | ↑ | 117±3.7$^{c}$ (69) | = |
| Sc1326 | 125±4.5$^{cde}$ (70) | 167±5.4$^{e}$ (87) | ↓ | 130±7.0$^{de}$ (72) | ↓ | 211±6.2$^{h}$ (113) | ↓ |
| Sc1340 | 121±6.8$^{bcd}$ (68) | 116±5.7$^{ab}$ (70) | ↑ | 115±4.5$^{abc}$ (63) | ↑ | 126±1.0$^{d}$ (78) | ↓ |
| Sc1591 | 131±2.5$^{ef}$ (72) | 133±8.5$^{c}$ (80) | = | 131±6.6$^{de}$ (73) | = | 119±4.8$^{c}$ (71) | ↑ |
| Sc1741 | 131±4.6$^{bc}$ (65) | 117±3.7$^{ab}$ (68) | ↑ | 116±9.1$^{abc}$ (68) | ↑ | 157±6.5$^{f}$ (93) | ↓ |
| Sc2306 | 152±9.0$^{g}$ (81) | 258±6.3$^{g}$ (122) | ↓ | 194±12.1$^{g}$ (93) | ↓ | 293±4.6$^{j}$ (140) | ↓ |
| Sc2621 | 137±9.0$^{f}$ (74) | 145±5.8$^{d}$ (88) | ↓ | 119±7.9$^{c}$ (62) | ↑ | 154±2.4$^{f}$ (94) | ↓ |
| BY4742 | 107±7.8$^{a}$ (56) | 137±1.4$^{cd}$ (81) | ↓ | 139±5.5$^{e}$ (77) | ↓ | 180±8.4$^{g}$ (102) | ↓ |
| Σ1278b | 192±8.2$^{h}$ (100) | 203±5.6$^{f}$ (112) | ↓ | 180±15.0$^{f}$ (94) | ↑ | 243±3.7$^{i}$ (129) | ↓ |

[a]Glucose

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336 **Fig. 1**

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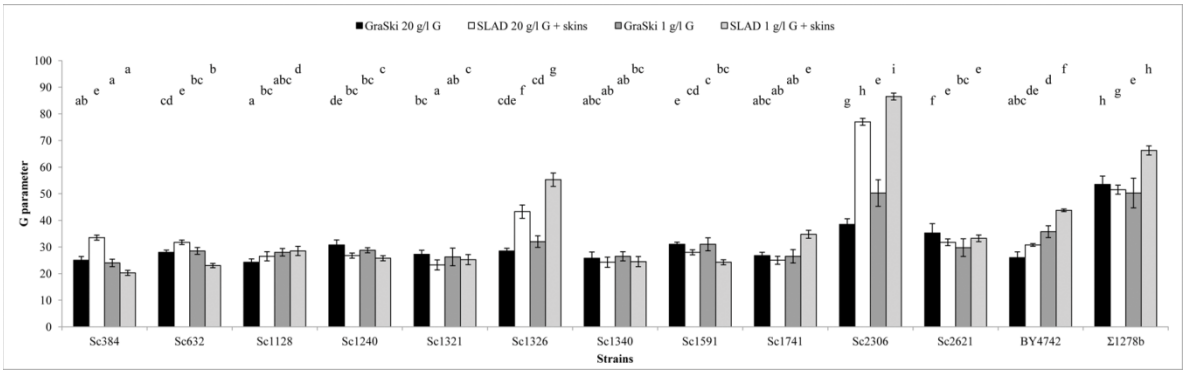


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340 **Fig. 2**

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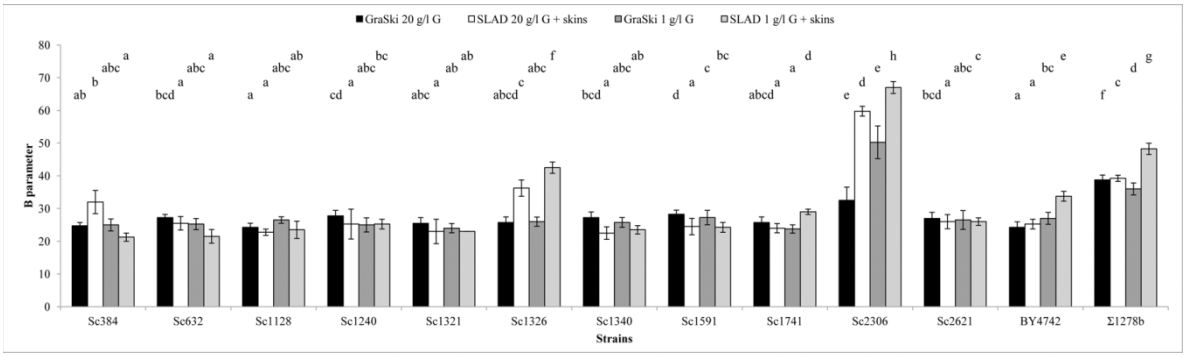


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344 **Fig. 3**