Using salinity to improve nutritional and market value of strawberries

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Abstract

Plants respond to salinity by producing antioxidants and osmolytes; some of these are nutritionally useful to humans (e.g. phenolics), or may improve the sensory quality of produce (e.g. sugars). For the current study, strawberries were irrigated with 0, 10, 20 or 40 mM NaCl, and the responses in antioxidants, phenolics, and flavour attributes were measured. A linear positive relationship was observed between salt stress and antioxidant concentrations, and the DPPH antioxidant assay responded more clearly than the FRAP assay. Phenolics were increased with statistical significance by salinity at all treatment levels, although trends differed between total phenolics and the subclasses anthocyanins and total flavonoids. It was seen that mild salinity (10 mM) with a low impact on yield could be used to increase antioxidants (6-10% above control) and total phenolics (11-16% above control). However differences between cultivars outweighed differences due to salinity in treatments below 20 mM NaCl. We found that sugars in strawberries (unlike other fruits) weren't increased by salinity treatment. The lack of sugar increase can be explained by suggesting that organic acids are more important osmolytes than sugars for strawberries under salinity. However it was also determined that the failure of strawberries to increase their soluble sugars during salt stress can be explained by the lean nutrient regimes used by researchers, which don't represent commercial production practices. Practically, mild salinity (≤20 mM) can be used to increase strawberry fruit value, but should be combined with an appropriate nutrient regime to avoid harm to flavour quality. The varieties Elsanta and Elsinore are both suitable for salt treatment, but Elsanta is better for antioxidant and phenolic production, as well as being more salt tolerant.

Keywords: strawberry, salinity, antioxidant, compatible solute, phenolic.

Introduction

Strawberry (*Fragaria × ananassa* Duch.) fruit is commonly considered to be a valuable nutritional resource of vitamins, minerals and phenolics (Giampieri et al*.,* 2014). However, both the sensory and nutritional qualities of strawberries are highly variable, with a large part of this variation being due to environmental factors (Terry et al*.,* 2009). With this in mind, salinity stress has previously been studied as a tool for improving the quality of strawberry fruit (Awang et al*.,* 1993*a*, *b*; Keutgen and Pawelzik 2007*a*). Depending on factors such as evaporation rates and rainfall, it is possible to use a limited amount of saline irrigation water without accumulating salt in the soil (Panta et al*.,* 2016).

Amongst the toxic effects of salinity are osmotic and oxidative stresses (Flowers et al*.,* 2015). While plants may counter increased osmolarity in the external environment by accumulating inorganic ions to a certain extent, (Shabala and Shabala 2011), it is also necessary to accumulate organic osmolytes, the so-called "compatible solutes" (Wyn Jones et al., 1976; Puniran-Hartley et al., 2014; Kameli and Losel 1995). Amongst the most abundant and widespread compatible

solutes are sugars, such as sucrose (Hughes et al*.,* 2013) and glucose (Kameli and Losel 1995). For this reason it may be expected, and has often been observed, (Wadsworth 1990; Johkan et al*.,* 2014), that plants subjected to salinity improve in sweetness. Other osmolytes which are important to the sensory quality of fruits include malic acid and citric acid (Li et al*.,* 2013; Keutgen and Pawelzik 2008*a*).

In addition to the obvious osmotic effect of salinity, there is also a well-known oxidative challenge to plants (Bose et al*.,* 2014). This is countered by mobilisation of enzymatic antioxidant systems (Kiani-Pouya, 2015; Turhan et al*.,* 2008), as well as non-enzymatic antioxidants. These include many of the compatible solutes, (including sugars), (Smirnoff and Cumbes 1989; Akashi et al*.,* 2001), and also phenolics, which in some cases are essential antioxidants (Agati et al*.,* 2012), and increase in response to salinity (Borochov-Neori et al*.,* 2013; Li et al*.,* 2013; Jamalian et al*.,* 2013; Neocleous and Vasilakakis 2009*a*). Given that phenolics have a profound impact on the nutritional value of fruits, an increase in phenolic antioxidants in response to salinity could be a major improvement in fruit

quality. Anthocyanins in particular are found relevant to topical health conditions (Poudyal et al., 2010), with ensuing public interest, (Courteney 2015; Lienard 2017).

Although salinity has been successful as a tool for improving sugar levels in other species of fruit (Sato et al., 2006; Li et al., 2013), most studies with strawberries have found that sugar concentrations or sweetness decrease under salinity (Saied et al., 2005; Keutgen and Pawelzik 2007b, 2008a, b; Kaya et al., 2002). In contrast, Khayyat et al., (2007) and Awang et al., (1993b) have shown that sugar levels are maintained or increased, and although these increases have been attributed solely to decreasing water content, the increase of sugar relative to dry matter indicates increased synthesis of sugars. Sugars increasing in response to salinity are also shown in Yaghubi et al., (2016).

All previous studies on the effect of salinity on flavour and sugar concentration in strawberry fruits used salinity treatments above 25 mM, which is enough to severely stress strawberry plants (Martinez Barroso and Alvarez 1997). It has been demonstrated in other fruits (grapes) that while higher salinities are detrimental to sugar concentrations, lower salinities may enhance them (Li et al., 2013). There is therefore a need to assess the effects of lower salinity levels on sugar concentration and flavour of strawberries. It is acknowledged that Neocleous and Vasilakakis (2009a) previously tested salinity as low as 10 mM on strawberries, but they didn't publish their data for sugar concentrations because they didn't obtain statistically significant results, nor did they conduct taste tests.

Aside from the potentially positive or negative effects salinity might have on the sensory quality of strawberries, effects on nutritional quality should be considered. Salinity produces strong increases in the antioxidant capacity of strawberry fruits, and in the concentration of phenolics (Neocleous and Vasilakakis 2009a; Keutgen and Pawelzik 2008b; Jamali et al., 2016). However, the loss of yield and quality that also follows salinity treatment in studies is commercially unacceptable. There is thus a need to investigate whether worthwhile improvements in nutritional quality can be produced while maintaining acceptable yield and sensory quality.

Since all of the above studies regarding yield and quality of strawberries under salinity examined treatments only above 25 mM sodium chloride, the current study examines a lower salinity range to determine if strawberries under milder stress would respond by increasing phenolics and antioxidants while upholding quality and yield.

Results

Fruit yield
In absence of salt, cv. Elsanta presented significantly bigger fruits (+30%) as compared to cv. Elsinore (Table 1). Salinity reduced fruit weight (Table 1) by 21% and 26% respectively for Elsanta and Elsinore under 40 mM NaCl (compared to control conditions). On the other hand, number of fruit per plant was significantly affected only by the cultivar (Table 1): cv. Elsinore presented a 58% greater number of fruits as compared to cv. Elsanta, resulting in Elsinore having 33% more yield than Elsanta under control conditions. Under salinity yield was

reduced more dramatically in Elsinore (reduction of 32% under 40 mM NaCl) as compared to Elsanta (reduction of 16 % under 40 mM NaCl) (Table 1).

Appearance, Aroma, Taste
Salinity resulted in decreased visual appeal of fruit ($P \leq 0.001$, Table 2), with decreases of 18% and 29% in cv. Elsanta and cv. Elsinore respectively under 40 mM NaCl. While Elsanta fruits were considered of "good" appearance under all salinity treatments (5.2–6.9), Elsinore was downgraded to "medium" at 20 and 40 mM NaCl (Table 2).

The highest scores for aroma were given to cv. Elsanta under control condition, which was categorised as "very good". Under 10 and 20 mM NaCl the score was downgraded to "good". Elsinore fruits scored "good" aroma under control, but also under 10 and 20 mM NaCl. Fruits of both cultivars under 40 mM NaCl were downgraded to "medium" (Table 2). Statistically, aroma was significantly ($P \leq 0.001$) impaired by increasing salt concentration in the growing media (Table 3) with a 39% and 30% decrease in cv. Elsanta and cv. Elsinore respectively at 40 mM NaCl as compared to control plants.

The taste of fruit of both cultivars was considered to be "good" with the exception of fruit of cv. Elsanta, which was "medium" (Table 4) under 40 mM NaCl. However, no significant differences in taste were revealed by the statistical analysis (Table 2). Further, there were no statistical differences found in the PCA analysis between any sensory attributes at either the level of cultivar or cultivar x salinity interaction (supplementary data).

pH, Total soluble solids (TSS), titratable acidity (TA), and TSS/TA ratio
pH and TSS were not affected to the extent of statistical significance either by cultivar or by salinity, however Elsanta did appear to maintain TSS under salinity better than Elsinore (Table 3). TA was also not statistically affected by salinity, but varied between cultivars, being 19-55% higher in Elsanta as compared to Elsinore (Table 3). Conversely, TSS/TA was consistently higher in Elsinore, with the difference between the varieties generally decreasing as salinity increased, and becoming statistically insignificant in the 40 mM treatment. Furthermore, while no salinity effects on TSS/TA could be observed in Elsanta, Elsinore displayed a clear reduction in TSS/TA at high salinity (30% under 40 mM), but little or no effect from 10 mM NaCl (Table 3).

Antioxidant Content
Fruits of cv. Elsanta under control conditions showed a significantly ($P \leq 0.001$) higher (+23% as compared to Elsinore) phenolic content (Table 4). In both cultivars, increasing salt treatment always increased phenolic content, which under 40 mM NaCl was 29% higher than control in Elsanta and 43% higher than control in Elsinore (Table 4).

Anthocyanin content was also improved by salt treatments in both cultivars (Table 4), and as with total phenolics it was improved to a greater extent in Elsanta (+23% at maximum, 40 mM NaCl) than Elsinore (+12% at maximum, 20 mM) (Table 4). However, anthocyanin content didn't always increase with salinity treatment in cv Elsinore, and in cv Elsanta the increases

weren't always statistically significant. Unlike total phenolic content, anthocyanin content was consistently higher (6-23%) in Elsinore than in Elsanta.

Total flavonoid content was increased by salinity in both varieties, but without clear trends being apparent. Nevertheless, in both cultivars the highest salinity treatment showed a significantly higher ($P \leq 0.001$) flavonoid content than the control. At all salinities Elsanta had a higher total flavonoid content than Elsinore.

Total antioxidant capacity showed a consistently increasing trend under salinity treatment in both cultivars, with both the DPPH and FRAP assays. The increases in antioxidant capacity were not always statistically significant, but both tests showed a significant difference between the highest salt treatment and the control, except for the FRAP assay of cv Elsinore, where the large variation in the FRAP measurements for the control prevented finding any statistically significant results. Elsanta had a higher antioxidant capacity than Elsinore under all treatments according to DPPH, and under all treatments except the control according to FRAP. However the differences were mostly not statistically significant.

A significant co-relation (Table 5) was found between DPPH value and phenolics, anthocyanin and total flavonoids in cv. Elsanta and with phenolics in cv. Elsinore. Similarly, FRAP assay was significantly co-related with total phenolics, anthocyanin and total flavonoids in cv. Elsanta (Table 5), but not with any of these measures in cv. Elsinore.

Discussion

Salinity effects on phenolics and on antioxidant capacity
An important finding in the current study is that it is possible to achieve improvements in the antioxidant capacity and phenolic content of strawberries using very low salinity levels that have only a small impact on yield. Previous authors had shown that antioxidants and phenolics could be increased with salinity treatments, (Keutgen and Pawelzik 2008*b*, 2007*a*; Khayyat et al., 2007) but their work used high salinity levels that had an unacceptable effect on yield.

The current study found that only 10 mM salinity was enough to produce an increase of total phenolics in both strawberry cultivars, and increases in flavonoids and anthocyanins that were statistically significant in at least one cultivar. 10 mM NaCl also produced increases in total antioxidant capacity in both cultivars by both measurements (DPPH and FRAP), and although the increases were not statistically significant the consistent trend for increasing antioxidant level with increasing salinity makes it likely that the observed increase for antioxidant capacity in fruits grown under 10 mM NaCl is real rather than random. If salinity had no effect and each treatment therefore had a 50% chance of producing a higher or lower result than the previous treatment, then there would be only a 4.2% chance of every treatment producing a higher antioxidant level than the previous one, (equivalent to a *P* value of 0.04%), and the chance of this happening in both varieties would be only 0.17%, (*P* < 0.005). This simple calculation is in fact conservative, because if the average result under control conditions is presumed to be close to the middle of a normal distribution curve, (for results in the absence of a treatment effect), then each successively higher result is further towards the tail of the curve, (meaning less and less than 50% chance of occurring), and becomes increasingly unlikely to happen in the absence of a treatment effect. Thus despite the lack of statistical significance in the antioxidant increases when assessed individually, the consistent increase found in both varieties makes it demonstrably improbable that they are due only to variance.

Yield loss in the 10 mM treatment was only 14% in Elsanta and 16% in Elsinore. Considering the emphasis on quality and willingness to pay for it by the consumers who buy strawberries most frequently (Wang et al., 2017), a premium price for strawberries with improved nutritional properties might realistically compensate for such yield losses.

Importantly, it was found that Elsanta under control or 10 mM NaCl produced more total phenolics and a greater antioxidant capacity than Elsinore under any salinity except 40 mM. Similar results appear in Keutgen and Pawelzik, (2007*a*), where the effect of cultivar was also larger than the effect of salinity on total phenolics in strawberries. Thus with low salinity treatment the effect of salinity is outweighed by the effect of cultivar, and it can't be claimed necessarily that one cultivar grown under saline conditions produces fruit with better antioxidant or phenolic properties than another grown without salinity. Thus, fruit grown under low salinity conditions (e.g. 10 mM NaCl) can only be said to have superior antioxidant and phenolic conditions if it is of the same variety as competing fruits grown without salinity, or of a variety known to generally produce more antioxidants and phenolics. In fact antioxidant production is a ubiquitous response to stress in plants, (Puniran-Hartley et al., 2014), and a wide variety of cultivation practices or growing conditions (Neocleous and Vasilakakis 2009*a*, *b*) could produce an increase in antioxidant levels. However, since the bulk of strawberries produced in a given region are usually produced by growers striving to avoid stressful conditions and very likely using the same varieties as each other, it is highly likely that a grower who deliberately used salinity stress really would have fruits with superior antioxidant and phenolic levels in comparison to his competitors'. Certainly it would be legitimate for a grower who produced a percentage of his fruit from salinity stressed plants while the bulk were produced under usual growing conditions to claim that the salinity stressed fruits had superior antioxidant and phenolic levels in comparison to the rest of his crop, and to seek a premium for those fruits.

Total phenolics increased with every salinity increase, in both varieties, (though not always with statistical significance), hinting that phenolics are an important part of the strawberry plant's response to salinity, and that phenolics can be maximised by salinity treatment. This was not true for individual classes of phenolics, (flavonoids and anthocyanins), so it may be that classes of phenolics differ in importance with the level of stress.

In assessing the antioxidant differences between varieties and treatments, it was found that total phenolics, FRAP and DPPH generally revealed similar trends. However, DPPH showed a stepwise increase in antioxidant effect whereas FRAP did not. FRAP is a convenient but poor antioxidant assay (Prior and Cao 2001). DPPH has also been found better than the β-carotene

bleaching method, (because the latter counts ascorbate as a pro-oxidant) (da Silva Pinto et al., 2008).

Influence of salinity on sensory quality of strawberries

Aroma and appearance scores were both decreased by salinity, but both varieties were able to maintain aroma and appearance of at least a "good" standard at 10 mM NaCl. The taste, (as well as the quantitative measurements TSS and TSS/TA), of fruits grown under 10 mM NaCl (which was enough to produce an increase in antioxidants and phenolics) was not statistically affected by salinity, and taste may even have been improved in Elsinore. In other fruit species, aroma compounds have been found to decrease under salinity even if taste compounds are increasing (Li et al., 2013).

Salinity did not have a statistically clear effect on TSS/TA in Elsanta, but there was an obvious trend towards decreasing TSS/TA in Elsinore. Given that Keutgen and Pawelzik (2008*b*, *a*) have found that amino acids and non-nitrogenous organic acids such as citric acid in salt-affected strawberries increase much more than sugars, a reduction in TSS/TA ratio would be expected. The TSS/TA ratio may also have been affected by changes in phenolics, (Talcott 2007), and a statistically significant relationship was found between TA and phenolics in the PCA (supplementary data).

All of the treatments in this study yielded fruit with TSS/TA occurring within the range that the Oregon Strawberry Commission (n.d.) deems to indicate flavour quality acceptable to the market (8.5-13.79 TSS/TA).

TSS/TA was not a strong indicator of taste test results in either Elsanta or Elsinore, perhaps because of the large variability (up to 20%) in TSS/TA. This variability came mainly from the TSS readings, as the variation in TA was less than 10% between replicates in every treatment group except one (the control treatment for Elsanta). However, despite the better repeatability for TA, it was also not a good predictor of taste test results. Taste test scores themselves did not vary by more than 11.5% within each group of replicates, meaning that the failure to match up with TSS/TA trends was not due to variability within the taste scores and can't be blamed on the subjective nature of taste tests. Thus it may be said that taste scores were not greatly influenced by changes in TA, and thus were probably driven by changes in TSS although the variability between replicates of this measurement prevented a clear connection. Other authors have also found that taste tests were less variable than analytical methods for sugars (Awang et al., 1993b).

In addition to the sugar/acid balance, the amino acid glutamine has previously been found to influence the flavour of salinity affected fruits of some species (Sato et al., 2006). This could also contribute to differences between quality assessments by TSS/TA vs taste in the current study.

In common with previous authors, (Keutgen and Pawelzik 2007*b*, 2008*a*; Saied et al., 2005; Kaya et al., 2002), the current study found that salinity didn't have a positive effect on sugar content in strawberries – unlike many other crops, which improve in sugar concentration and taste (Wadsworth 1990; Li et al., 2013; Johkan et al., 2014; Sgherri et al., 2008). This is contrary to the expectation that synthesis of soluble sugars will be increased to help with osmotic adjustment and the many other roles of compatible solutes during salinity stress.

A possible explanation is that strawberries might emphasise amino acids and organic acids rather than soluble sugars as compatible solutes, as is supported by the finding of other authors (Garriga et al., 2015; Keutgen and Pawelzik 2008*b, a*) that amino acids and non-nitrogenous organic acids in strawberry fruits increased in response to salinity while soluble sugars did not. Using sugars as osmolytes makes sense for healthy plants, which have abundant stored starch that can easily be broken into sugars to increase osmolarity, without great expenditure. However in plants that are more severely stressed and have little stored starch, it might not be sensible to make sugars specifically for use as compatible solutes, if other organic solutes, (such as amino acids), give better value as compatible solutes, (with roles as antioxidants, osmolytes, and protein stabilisers).

A less esoteric explanation is that strawberries grown for the current study and significant prior authors on the topic (Keutgen and Pawelzik 2008*b, a*, 2007*a, b*; Saied et al., 2005), did not have a sufficient nutrient supply to maintain photosynthesis (and thus soluble sugar synthesis) during salinity stress. It has already been demonstrated, (Kaya et al., 2002; Ferreira et al., 2019), that soluble sugar levels in strawberries under NaCl stress can be maintained close to control levels when supplementary nutrition (calcium) is provided, and other authors have shown that plants stressed by high EC nutrient solutions without NaCl maintain or increase soluble sugar content in fruits (Sonneveld and Welles 1988). Thus there are grounds to consider that poor nutrition might be a factor if NaCl stressed fruits don't show the expected increase in sugar concentration.

Poor nutrition as an explanation for the lack of upregulated sugar synthesis is supported by reference to Awang et al., (1993), Khayyat et al., (2007) and Yaghubi et al., (2016) who all found that soluble sugars in strawberries were increased by salinity treatment, and who all used a more liberal nutrient supply (i.e. nutrient always present in the irrigation water) than the current study and significant prior authors who have found sugar levels not increasing under salinity (Keutgen and Pawelzik 2008*a*, 2007*b*; Saied et al., 2005). The current study and the works of the aforementioned authors who likewise found decreasing sugar levels were all based on plants grown in pot culture with fertigation only once or twice a week. In the present case the nutrient solution was applied once a week and had an EC of 1.75 dS/m, which is rather less than Awang et al., (1993*b*), (who used 2.5 mS/cm), but comparable with Keutgen and Pawelzik (2007*a*) - upon which is based the method of Keutgen and Pawelzik (2008*b*) and presumably also Keutgen and Pawelzik (2008*a*) and (2007*b*). The levels of nutrient supplied in Keutgen and Pawelzik (2007*a, b*, 2008*a, b*), and Saied et al., (2005) as well as the current study would be considered substandard by commercial growers. For example, the Department of Food and Agriculture (Western Australia) recommends that strawberries grown on the poor sandy soils of the Perth coastal plain be fertigated 1-4 times a day with a nutrient solution of "around 2.2 mS" including the EC of the bore water, which might be around 0.5 mS (https://www.agric.wa.gov.au/strawberries/fertilising-your-

strawberry-crop viewed 24 April 2015). Assuming a planting density within the range recommended by the department (https://www.agric.wa.gov.au/strawberries/strawberries-growing-crop viewed 24 April 2015), this would amount to 0.14-0.17 g of Ca per plant per week. Keutgen and Pawelzik applied only 0.04 g of Ca per week, and Saied et al., (2005) applied even less. Never the less, before concluding that failure to find upregulated soluble sugar production is merely a matter of inadequate plant nutrition, it should be noted that Kaya et al., (2002) also found that sugar levels were negatively impacted by salinity, despite fertigating with up to 500 mL of nutrient solution per plant per day. However given that the plants were in washed sand (i.e. a media with low capacity for holding water and nutrient), and outdoors, this rate of fertigation would certainly have been inadequate in some weather (Higgs and Jones 1989), such that the salt stress would have been compounded by drought stress. Further, Kaya et al., (2002) used particularly salt sensitive cultivars (Yildiz et al., 2008), whereas at least one of the studies that found sugar levels improved by salinity (Awang et al., 1993*b*) used a relatively tolerant cultivar (Yildiz et al., 2008). Other factors, such as the possibility of heat stress caused by the black covers on the pots, or the balance of the nutrient solution (which was quite different to Awang et al., 1993*b*) might also have played a part in the results of Kaya et al., (2002).

Despite these limitations, Kaya et al., (2002) demonstrated that supplementary nutrients (calcium sulphate) allowed strawberries under salinity to maintain sugar levels close to control levels. Khayyat et al., (2007) also found that with supplementary nutrients strawberries could keep sugar levels at control levels or better during salinity stress. These findings lend strength to the supposition that the difference between studies finding upward regulation versus loss of sugars in strawberries under salinity may be a matter of liberal vs lean nutrient regimes.

This leads to the important summation that in all likelihood the quality improvements (antioxidants and phenolics) that the current study has found can be brought by mild salinity, may yet be combined with salinity-induced taste enhancements, given adequate crop nutrition and appropriate cultivar choice. The failure of previous authors, and the current study, to produce the increase of sweetness that is usually observed in salinity stressed fruits of other species might be due to lean nutrient regimes that don't represent commercial cropping systems.

Influence of salinity on strawberry yield
The decline in yield that salinity induced was steeper in Elsinore than Elsanta, but both cultivars demonstrated the supposition that yield loss could be minimised by using lower salinity treatments to induce phenolics and antioxidants. For comparison, the mildest salinity treatment found in the literature of previous authors examining the effect of salinity on antioxidants in strawberries was 30 mM, and precipitated a 29% reduction in yield for Elsanta (Saied et al., 2005); whereas in the current study the mild 10 mM treatment only reduced yield by 14% in Elsanta.

Effect of cultivar choice on strawberry production under salinity
Although Elsinore suffered a greater loss of yield from salinity, its yield remained higher than that of Elsanta in all treatments. Elsinore also improved in taste (although not with statistical significance) whereas Elsanta deteriorated, such that under salinity treatment the flavour of Elsinore was always as good as or better than that of Elsanta. On the other hand, Elsanta was a better cultivar for antioxidant activity and phenolic content under all salinity levels, and even under control conditions it produced similar phenolic content and antioxidant activity (by DPPH) to Elsinore under 20 mM NaCl.

Thus for the commercially important qualities yield and taste, Elsinore would be preferred over Elsanta for strawberry production under salinity, but the fruits thus produced couldn't necessarily be claimed to be superior to Elsanta produced under non-saline conditions in terms of antioxidants and phenolics unless the salinity level exceeded 20 mM.

The comparatively high antioxidant capacity of Elsanta was previously observed by Keutgen and Pawelzik (2007*a*) when they compared it to the relatively salt tolerant cultivar Korona, (despite the fact that Korona had greater phenolics). Although the difference between Elsanta and the comparison variety (either Elsinore or Korona) wasn't statistically certain in either the current study or in Keutgen and Pawelzik (2007*a*), the fact that Elsanta had equal or greater antioxidant capacity under every salinity treatment in two separate studies, and by different assays (FRAP and DPPH), (with the exception of the FRAP measurement for control conditions in the current study), greatly increases the probability that Elsanta is genuinely higher in antioxidants than other cultivars.

Contribution of antioxidants (phenolics and compatible solutes) to salinity tolerance mechanisms in strawberries
Anthocyanins and total phenolics, (but not flavonoids), each responded to progressive increases in salinity, in both cultivars. Antioxidant capacity increased at the same time. The anthocyanin and total phenolic concentrations, and the antioxidant capacity, of Elsanta could therefore be put forward as explanations for the greater salinity tolerance of Elsanta. Using Orsini et al., (2012) and Keutgen and Pawelzik (2007*a*) along with the current study, it is possible to rank the studied strawberry cultivars for salinity tolerance in the order Korona>Elsanta>Elsinore, and observe that this hierarchy is also true for phenolic and anthocyanin concentrations under salinity treatments, but not under control conditions. A supposition can therefore be made that inducible anthocyanin and total phenolic contents, but perhaps not constitutive concentrations, are significant salinity tolerance mechanisms in strawberries. On the other hand, the higher inducible concentrations in more tolerant varieties might be the consequence of synthesis pathways being better protected by other salinity tolerance mechanisms, and it must be noted that Keutgen and Pawelzik (2007*a*) didn't find that Korona had a superior antioxidant capacity to Elsanta and yet was more salt tolerant. Further, oxidative damage (ascertained by analysis of malondialdehyde) does not co-relate well with susceptibility of strawberry varieties to salinity (Yildiz et al., 2008). It seems

Table 1. Influence of salinity treatments (0, 10, 20 and 40 mM NaCl) on fruit weight, fruit number, and yield per square metre.

Cultivar	NaCl (mM)	Fruit weight g/fruit	Fruit number fruits/plant	Fruit yield kg/m^2
Elsanta	0	18.3 ± 0.49 a	9.9 ± 0.18 b	1.49 ± 0.098 c
	10	15.7 ± 0.56 b	9.5 ± 0.84 b	1.28 ± 0.103 d
	20	15.4 ± 0.84 b	9.6 ± 0.37 b	1.27 ± 0.115 d
	40	14.5 ± 1.02 bc	10.0 ± 0.67 b	1.24 ± 0.072 d
Elsinore	0	14.1 ± 1.71 bc	16.7 ± 0.38 a	1.98 ± 0.130 a
	10	13.3 ± 1.51 cd	14.8 ± 0.16 a	1.66 ± 0.112 b
	20	12.1 ± 0.84 de	17.4 ± 0.50 a	1.84 ± 0.115 ab
	40	10.5 ± 2.94 e	15.7 ± 1.17 a	1.35 ± 0.109 cd
Cultivar (C)		***	***	*
Salt (S)		***	ns	***
C x S		ns	ns	ns

Mean values ± SD (n=10); results marked with the same lowercase letter are not statistically different to each other, (standard of *P*≤0.05). Only results within the same column are compared, (including both varieties). ns = non-significant differences; * = significant differences at *P*≤0.05; ** = significant differences at *P*≤0.01; *** = significant differences at *P*≤0.001.

Table 2. Influence of salinity treatments (0, 10, 20 and 40 mM NaCl) on sensory panel test of fresh fruit of two strawberry cultivars, Elsanta and Elsinore.

Cultivar	NaCl (mM)	Appearance	Aroma	Taste
Elsanta	0	6.3 ± 0.42 ab	8.0 ± 0.21 a	6.2 ± 0.61 a
	10	6.9 ± 0.35 a	5.2 ± 0.76 bc	6.0 ± 0.49 a
	20	5.6 ± 0.34 bc	5.4 ± 0.60 bc	5.7 ± 0.60 a
	40	5.2 ± 0.25 cd	4.9 ± 0.55 c	4.8 ± 0.55 a
Elsinore	0	6.2 ± 0.29 ab	6.6 ± 0.72 ab	5.1 ± 0.50 a
	10	6.6 ± 0.31 a	5.9 ± 0.46 bc	6.0 ± 0.65 a
	20	4.9 ± 0.38 cd	5.3 ± 0.67 bc	6.3 ± 0.40 a
	40	4.5 ± 0.37 d	4.6 ± 0.79 c	5.1 ± 0.57 a
Salt (S)		***	***	ns
Cultivar (C)		ns	ns	ns
S × C		ns	ns	ns

Mean values ± SD (n=10); results marked with the same lowercase letter are not statistically different to each other, (standard of *P*≤0.05). Only results within the same column are compared, (including both varieties). ns = nonsignificant differences; * = significant differences at *P*≤0.05; ** = significant differences at *P*≤0.01; *** = significant differences at *P*≤0.001

Table 3. pH, total soluble solids (TSS), titratable acid (TA), and TSS/TA ratio of two strawberry cultivars, Elsanta and Elsinore, grown in different NaCl concentrations (0, 10, 20 and 40 mM NaCl).

Cultivar	NaCl (mM)	pH	TSS (%)	TA (g/100mL)	TSS/TA
Elsanta	0	3.59 ± 0.096 a	9.9 ± 0.53 a	0.88 ± 0.109 a	11.4 ± 0.91 c
	10	3.51 ± 0.017 a	10.2 ± 1.37 a	0.99 ± 0.041 a	10.3 ± 1.45 c
	20	3.55 ± 0.055 a	10.0 ± 1.06 a	0.95 ± 0.051 a	10.6 ± 1.11 c
	40	3.70 ± 0.074 a	9.1 ± 1.59 a	0.81 ± 0.053 ab	11.3 ± 1.94 c
Elsinore	0	3.54 ± 0.156 a	10.5 ± 0.79 a	0.64 ± 0.038 b	16.6 ± 2.01 a
	10	3.73 ± 0.116 a	10.3 ± 0.93 a	0.64 ± 0.060 b	16.1 ± 0.60 ab
	20	3.67 ± 0.084 a	8.2 ± 1.55 a	0.67 ± 0.043 b	12.3 ± 2.5 abc
	40	3.72 ± 0.066 a	8.0 ± 0.31 a	0.68 ± 0.015 b	11.7 ± 0.24 bc
Salt (S)		ns	ns	ns	ns
Cultivar (C)		ns	ns	***	***
S x C		ns	ns	ns	*

Mean values ± SD (n = 6); results marked with the same lowercase letter are not statistically different to each other, (standard of *P*≤0.05). Only results within the same column are compared, (including both varieties). ns = nonsignificant differences; * = significant differences at *P*≤0.05; ** = significant differences at *P*≤0.01; *** = significant differences at *P*≤0.001

Table 4. Influence of salinity treatments (0, 10, 20 and 40 mM NaCl) on fruit content of total phenolics, anthocyanins and flavonoids, and antioxidant capacity by DPPH and FRAP assays of two strawberry cultivars, Elsanta and Elsinore.

Cultivar	NaCl [1]	Phenolics	Anthocyanin	Flavonoids	DPPH [2]	FRAP [3]
	(mM)	(mg GA)	(mg pg-3-glu eq.)	(mg CE)	(μM Trolox)	(mM Fe^{2+})
Elsanta	0	208 ± 2.5 c	20.9 ± 0.16 e	18.3 ± 0.38 b	21.6 ± 1.14 bc	0.73 ± 0.033 c
	10	232 ± 11.6 b	22.7 ± 0.69 de	20.3 ± 0.58 a	23.8 ± 0.91 ab	0.79 ± 0.031 abc
	20	245 ± 2.5 b	23.5 ± 0.87 cd	20.2 ± 0.19 a	24.3 ± 0.38 ab	0.85 ± 0.000 a
	40	269 ± 3.3 a	25.8 ± 1.21 b	20.1 ± 0.31 a	26.3 ± 1.10 a	0.85 ± 0.036 a
Elsinore	0	170 ± 14.3 d	25.8 ± 1.82 b	16.1 ± 0.26 c	19.1 ± 1.10 c	0.77 ± 0.100 bc
	10	197 ± 6.00 c	25.3 ± 1.54 bc	14.7 ± 0.98 c	20.3 ± 1.59 c	0.79 ± 0.033 abc
	20	214 ± 11.6 c	29.0 ± 0.67 a	15.9 ± 0.40 c	21.4 ± 2.89 c	0.79 ± 0.060 abc
	40	244 ± 4.6 b	27.3 ± 0.90 ab	18.7 ± 0.54 ab	26.1 ± 2.75 a	0.83 ± 0.012 ab
Salt (S)		***	***	***	***	**
Cultivar (C)		***	***	***	ns	ns
S x C		ns	**	*	*	ns

Results are expressed per 100g of fresh weight. Mean values ± SD (n = 6); results marked with the same lowercase letter are not statistically different to each other, (standard of $P \leq 0.05$). Only results within the same column are compared, (including both varieties). ns = nonsignificant differences; * = significant differences at $P \leq 0.05$; ** = significant differences at $P \leq 0.01$; *** = significant differences at $P \leq 0.001$.

Table 5. Pearson's correlation coefficients of antioxidant parameters for two strawberry cultivars under different salt concentrations.

Cultivar	Variables	Phenolics	Anthocyanin	Flavonoids
Elsanta	Salt	0.93[**]	0.82[**]	0.72[**]
	DPPH	0.77[**]	0.65[*]	0.68[*]
	FRAP	0.71[**]	0.79[**]	0.77[**]
Elsanta	Salt	0.95[**]	0.39	0.62[*]
	DPPH	0.70[*]	0.41	0.52
	FRAP	0.25	0.41	0.089

TPC= total phenolic content, TAC = total anthocyanin content, TFC= total flavonoids content, S= salt treatments, * = significant differences at $P \leq 0.05$; ** = significant differences at $P \leq 0.01$; No asterisk = no significant difference.

therefore that antioxidant mechanisms alone are not the most effective tools for salinity resistance in strawberries.

Unlike phenolics, soluble sugars did not respond positively to salinity in either cultivar, (assuming that TSS refers primarily to sugars). Sugars are usually amongst the most prolific compatible solutes, which begs the question, how was osmotic adjustment achieved without them? While inorganic ions may contribute the bulk of total osmolarity in plant tissues, the toxicity of inorganic ions means that compatible solutes are needed to adjust to osmotic changes when inorganic ions reach the threshold for toxicity, at least in the cytosol (as opposed to the vacuole) (Puniran-Hartley et al., 2014; Wyn-Jones et al., 1976; Kameli and Losel 1995). Thus if soluble sugars weren't used to achieve osmotic adjustment, amino acids and non-nitrogenous organic acids (e.g. citric acid) probably had to be used instead. This supposition is supported by the work of Keutgen and Pawelzik (2008a), who found that while soluble carbohydrates decreased when strawberry fruits were subjected to salinity, amino acids and non-nitrogenous organic acids increased. The current study's finding that TSS/TA decreased with salinity is also consistent with the hypothesis that organic (including amino) acids are more essential than sugars as compatible solutes for strawberries. In grapes it has also been shown that under relatively strong salinity stress organic acids increased while sugars decreased, even though at milder salinities sugars were able to increase in response (Li et al., 2013).

As well as being able to contribute to osmotic adjustment, amino acids can be important antioxidants in plants (Akashi et al., 2001; Cuin and Shabala 2007). While in the current study the increase of antioxidant capacity associated with salinity treatment, and the superior antioxidant capacity of Elsanta vs Elsinore, could be explained by corresponding increases in phenolics, in other studies this was not the case. Thus, caution should be used in explaining the antioxidant results in the current study by relation to phenolics. In a previous study (Keutgen and Pawelzik 2007a) the superior antioxidant capacity of Elsanta in comparison to another cultivar couldn't be explained by analysis of total phenolics, nor by anthocyanins, superoxide dismutase, α-tocopherol, ascorbic acid, or glutathione. Although Keutgen and Pawelzik (2007a) did not measure amino acids, the failure of other antioxidant mechanisms to explain the difference between cultivars leaves amino acids as a probable explanation, given that soluble sugars may also be ruled out on the basis that Keutgen and Pawelzik (2008a) have shown that they decrease under the regime used by Keutgen and Pawelzik (2007a). Thus by analysis of the data of Keutgen and Pawelzik (2008a, 2007a) it can be

deduced that amino acids are highly likely to be contributing to antioxidant capacity, even though the differences between cultivars and salt treatments in the current study can be adequately explained by changes in phenolics.

The observation that phenolics increased with salt stress, while sugars decreased, leads to a supposition that phenolics are more cost effective and essential antioxidants than sugars in strawberries under salinity. The effect of phenolics on antioxidant capacity may not be only due to total phenolic content, as different cultivars showed different behaviours in synthesis of anthocyanins and total flavonoids. Elsanta, which had the greater antioxidant capacity, also appears to have a greater constitutive synthesis of total flavonoids, which changed relatively little under salinity. Its concentration of anthocyanins on the other hand, while lower than that in Elsinore, was more responsive to salinity. It can therefore be suggested that the higher antioxidant capacity of Elsanta could be due to a higher constitutive synthesis of flavonoids other than anthocyanins, and a greater proportional increase of anthocyanins in response to salinity.

Materials and methods

Plant Material and Growth Conditions

The cultivars chosen had differing salinity tolerance, with Elsanta being more tolerant than Elsinore (Orsini et al., 2012), although sensitive relative to other cultivars (Keutgen and Pawelzik 2008*b*, 2007*a*). The experiment was conducted in a glasshouse at the experimental farm of the University of Bologna, located in *Ozzano dell'Emilia* (44º26'38 N, 11º26'18'' E, 98 metres above sea level). Plantlets of similar height and diameter were transplanted on the 16th February 2010 into plastic pots of 5 L volume (1 plant per pot), filled with a mix of commercial growing media and pumice (2:1 v/v). Pots were placed on benches at a density of approximately 9 plants/m^2. Plants were irrigated automatically three times a day and fertigation was carried out once a week by adding nutrients to the irrigation water at the following concentrations: $N\text{-}NO_3$ = 6.0 mM; $N\text{-}NH^4$ = 1.0 mM; PO_4^{3-} = 3.0 mM; K^+ = 4.0 mM; SO_4^{2-} = 7.0 mM; Ca^{2+} = 5.0 mM; Mg^{2+} = 4.0 mM; microelements in traces, at a final electrical conductivity (EC) of 2.2 dS/m including the background EC of the irrigation water, (calculated according to University of California 2019). Before salt stress was started, stolons were removed to conserve energy for fruiting.

Treatments and Experimental Design

Eight treatments, derived by the factorial combination of 2 cultivars (Elsanta and Elsinore) and 4 NaCl concentrations in the irrigation water (0, 10, 20 and 40 mM added NaCl), were compared. The experiment was laid out on 4 greenhouse benches, with one salt treatment assigned to each bench. Each bench was divided into 6 replicate plots; 3 for each cultivar, arranged so that no plot was adjacent to another of the same cultivar. There were 8 plants in each plot, so each bench, (or salt treatment), had 48 plants, (24 of each cultivar) (Supplementary Figure 2). The salt stress treatment was initiated on 1st April (44 days after transplanting, when the plants had 6–7 leaves), by irrigating plants with water having no added NaCl (henceforth called control or 0 mM NaCl), 10 mM added NaCl, 20 mM added NaCl, or 40 mM added NaCl. The EC of the irrigation water with no added NaCl was 0.45 dS/m, and the EC of the water after salt addition was calculated to be approximately 1.42 dS/m for the 10 mM NaCl treatment, 2.40 dS/m for the 20 mM treatment, and 4.35 dS/m for the 40 mM treatment (University of California 2019). When the weekly fertigation took place, the same amounts of NaCl were added to the fertigation water as to the plain irrigation water. This irrigation regime was maintained until the end of the experiment. Fruit-setting started on the 15th April (14 days after stress treatment initiation). Fruit of all plants was harvested manually at full maturity on four dates: 5th, 10th, 17th, and 27th May (corresponding to 34, 39, 46 and 56 days after commencing treatment). Fruit number and weight were determined at the end of the experiment. For most measurements the reported results are averaged across harvest dates, but sensory panel tests used only fruits from the harvests of the 10th and 17th of May.

Sensory Panel Test

Strawberry fruits were harvested ripe and offered immediately for sensory evaluation. The sensory panel test was done by 10 panellists, and was conducted according to the method of Azodanlou et al., (2003). Encoded strawberry fruit boxes were randomly posted on tables under well-controlled conditions with adequate distance between boxes to prevent aroma interference. Panellists drank water between samples. The panellists were asked to rate the following sensory attributes: appearance, aroma, taste. Evaluations of fruit appearance, aroma, and taste were based on the quality assessment model of Azodanlou et al., (2003). The model scores fruit on a scale of 1-9, divided into three quality levels with average values of 4.5 (range 4–5, "medium"), 6 (range 5–7, "good") and 8.5 (range 8–9, "very good").

Titratable Acid (TA), pH, Total Soluble Solids (TSS)

TA was determined by potentiometric titration (Giusti and Wrolstad 2001a). Titratable extract was obtained from 100-200 g of fresh fruit blended at 100 rpm for 1 minute and then centrifuged for 5 minutes at 5000 rpm (at room temperature). Five millilitres of the supernatant was pipetted into a 250 mL beaker and titrated with 0.1 M NaOH solution to the end point of pH 8.2 while stirring with a magnetic stirrer. TA is reported in terms of citric acid equivalent.

For pH, 5 mL of supernatant from blended strawberry fruit was measured by pH meter (GLP 22 CRISON, Barcelona, Spain).

The total soluble solids (TSS) content was determined using a refractometer (PCE, Southampton, UK).

Preparation of Extracts for Determining Phenolics and Antioxidant Activities

The frozen samples were thawed at room temperature and 10g of each sample was homogenised with 50 mL of methanol/H_2O/acetone (60/30/10; v/v/v) (Hartmann et al., 2008); after which the mixture was centrifuged at 10000 rpm for 10 minutes and the supernatant was collected. The extraction was repeated once and the combined supernatants

were used for the determination of total phenolics, flavonoids, anthocyanin, and antioxidative capacity.

Total Phenolic Content
Total phenolic content (TPC) was determined by Folin-Ciocalteau colourimetry (Giusti and Wrolstad 2002). All samples were measured in duplicate and the total phenolic content was expressed as gallic acid equivalent (GAE) in mg/100g of fresh weight (mg of GAE/100g FW).

Total Flavonoids Content
Total flavonoids (TFC) content was determined by aluminium chloride colourimetric assay (Zhishen et al., 1999). The results were expressed as mg of catechin equivalents (CE)/100g of fruit fresh weight. Samples were measured in duplicate.

Total Anthocyanin Content
The total anthocyanin content (TAC) was determined by the pH-differential method (Giusti and Wrolstad 2001b, validated by Tonutare et al., 2014). The total anthocyanin content was calculated according to Hartmann et al., (2008), and expressed in mg pelargonidin-3-glucoside equivalent per 100g of fruit fresh weight. 2 further replicates were made and measured.

Ferric Reducing Antioxidant Power (FRAP) assay
Total antioxidant activity was measured by a ferric reducing antioxidant power (FRAP) assay according to the method of Benzie and Strain (1999), as modified by Aaby et al., (2007). FRAP values were expressed as mM of Fe (II) per 100 g of FW.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay
2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was done according to Alamanni and Cosu (2004) with the modifications by Hartmman et al., (2008). Antioxidant capacity was expressed in µM/L of trolox equivalents per 100g of FW.

Statistical Analysis
Statistical analysis was performed using ANOVA and LSD, calculated by SPSS statistical programme (SPSS, Chicago, IL, USA). Two-way ANOVA was used, with the two factors being salinity, (with 4 levels: 0, 10, 20, 40 mM), and cultivar, (with 2 levels: cv Elsanta and cv Elsinore). Furthermore, principal component analysis (PCA) was performed with Statistica software version 7.1 (StatSoft, Tulsa, Oklahoma, USA).

It is acknowledged that the chance of falsely finding a statistical difference between treatments, (a so-called Type 1 error), accumulates with the number of comparisons made, and that the statistical methods chosen for the current study don't take strong measures to lessen the rate of this error. The methods that are usually used to lessen the chance of a Type 1 error, (such as Tukey's HSD), do so at the cost of an exacerbated risk of failure to identify truly significant treatment differences, (so-called Type 2 error). In most cases this results in a greater over-all chance of making a false judgement (Saville 1990), so such methods should not be used where they are not necessary. Methods that strictly control the risk of a Type 1 error are generally not called for unless a single false judgement within a multiple comparison will invalidate all other comparisons (Benjamini and Hochberg 1995), which is not the case in the current study.

Conclusion

It can be seen that quality attributes of strawberries (antioxidant capacity, anthocyanin content, total flavonoids and total phenolics) are improved by using low salinities that have minimal impact on yield (around 15% yield loss, compared with about 30% or more in previous studies). Flavour of fruits treated with low salinities doesn't suffer statistically significant deterioration.

In common with previous authors, it was found that sugar content of strawberry fruits was negatively impacted by salinity treatments, even with the low salinity treatments that distinguish the current study from others. However, by reviewing the data of other authors it was found that observations of decreasing sugar content in strawberries under salinity are associated with lean nutrient regimes, and subsequently it is proposed that this is the chief explanation for conflicting reports by different authors.

Recommendation is made for further studies with low level salinity stress and alternative nutrient regimes in commercially realistic systems to optimise the production of highly flavoured fruits with enhanced antioxidant and phenolic properties.

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