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# Uptake and Translocation of Foliar-Applied L-Proline in Sweet Cherry (*Prunus avium* L.)

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**Abstract:** Foliar application of nitrogen (N) may supplement soil-applied N in sweet cherry orchards. The proteinogenic amino acid L-proline is a potential source of organic N. However, little is understood about its uptake and effects on fruit quality. In this study, $^{15}$N-labelled L-proline was spray-applied to branches of the cultivar 'Lapins' either pre- or post-harvest. Leaves, fruit, and whole branches were sampled to investigate the uptake and allocation of foliar-applied N. Both treatments resulted in elevated $^{15}$N levels in leaves, with N derived from proline (%NDP) comprising 0.22% and 0.45% after pre- and post-harvest applications, respectively. The fruit was a sink for pre-harvest L-proline, with the highest %NDP in the pedicel (0.21%), followed by the skin (0.17%) and flesh (0.12%). Quality outcomes of smaller, darker fruit with lower stem retention indicate advanced maturity following L-proline application. Both pre- and post-harvest treatments resulted in the recovery of $^{15}$N in branches at late dormancy, with %NDP in bark (0.12%), buds (0.15%), and wood (0.02%) of the post-harvest treatment twice as high compared with those from the pre-harvest treatment. This study demonstrates proof of concept of the uptake of L-proline into the leaves of sweet cherry plants and translocation into the fruit and storage organs of the branch.

**Keywords:** $^{15}$N; anthocyanins; foliar fertiliser; fruit quality; pre-harvest; post-harvest



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## 1. Introduction

Nitrogen is an essential macro-nutrient for deciduous fruit trees and can have a significant influence on tree development and fruit quality of sweet cherry (*Prunus avium* L.) [1]. Plants are able to take up N directly from the soil, most readily in inorganic but also in organic forms [2]. In commercial sweet cherry orchards, N is generally applied to the soil with irrigation water as nitrate or ammonium, commencing after full bloom. However, inorganic forms of N, particularly nitrate, are subject to loss from the soil through leaching [3] and/or nitrous oxide emissions [4,5].

Being a deciduous tree, sweet cherry remobilises N from storage reserves for early spring growth and flowering [6]. Thereafter, N from external sources is utilised for early fruit development and vegetative growth [7]. Post-harvest, N application is a common strategy in commercial orchards to build up storage reserves for the following season. However, the uptake of soil-applied N can be limited, especially within the season when applied post-harvest [8,9].

Direct foliar application of plant-available forms of N has potential to supplement soil-applied N. The absorption process of nutrients by leaves is passive, and uptake rates are influenced by the barrier function of the leaf surface and the nutrient concentration gradient [10]. Depending on the properties of the foliar nutrient, penetration of the leaf surface can occur via both the stomatal cavity and the cuticular surface [11]. Urea, a water soluble and non-polar form of N, has been shown to be absorbed by sweet cherry leaves, translocated within the tree and remobilised in the following season, hence functioning as a source of N when applied post-harvest [12].

Furuya and Umemiya [13] showed that amino acids are absorbed by fruit tree leaves with absorption rates negatively correlated with the molecular weight of the amino acid. The proteogenic amino acid L-proline is considered an important component of cell wall proteins and plays a role in the regulation of protein biosynthesis [14]. With its low molecular weight of 115.13 g $mol^{-1}$, non-polarity, and water solubility, L-proline is a promising candidate as an organic foliar N source. Foliar application of L-proline has been shown to have positive effects on fruit quality, such as increased sugar content in Japanese pears [15] and increased size, weight, and sugar content in oranges [16] and pomegranates [17]. To our knowledge, the effect of foliar L-proline application on sweet cherry fruit quality has not been evaluated.

Although the potential of foliar spray application has been demonstrated, there remains uncertainty around whether L-proline is taken up by cherry leaves, followed by translocation into other parts of the tree. Stable isotope-labelled N sources ($^{15}N$) represent a research tool to investigate this in a proof-of-concept scenario. There is evidence that inorganic $^{15}N$-labelled urea is taken up by cherry leaves [12], and we hypothesise that organic sources such as $^{15}N$-labelled L-proline will also be taken up, translocated, and incorporated into the tissues of sweet cherry trees. Therefore, the objectives of this study were to (1) measure the uptake of spray-applied $^{15}N$-labelled L-proline into cherry leaves; (2) investigate the translocation to fruit and branch tissues; and (3) compare the recovered $^{15}N$ from L-proline of pre- and post-harvest application. In addition, we investigated the effect of pre-harvest application of this alternative N source on selected quality parameters in sweet cherry fruit.

## 2. Materials and Methods

### *2.1. Study Site*

A trial was conducted in a commercial sweet cherry orchard at Rosegarland in Tasmania, Australia (42.71° S, 146.94° S, 130 m above sea level) during the 2018/2019 season. This region has a cool temperate climate with an average annual rainfall of 572 mm [4]. Trees were mature 7-year-old 'Lapins' on Colt rootstock trained to a Kym Green Bush system [18], with row spacing of 4.5 m and tree spacing of 1.7 m. The block was under bird exclusion netting, and commercial orchard management practices were maintained throughout the duration of the trial with drip irrigation providing water and nutrients to the trees. A total of 20 kg N $ha^{-1}$ was applied as nitrate via fertigation and foliar application before trial commencement; post-harvest total N input was 60 kg $ha^{-1}$, applied as urea via foliar application at 17, 20, and 23 weeks after full bloom (WAFB; 5 kg N each) and fertigation at 22, 23, and 24 WAFB (15 kg N each).

### *2.2. Trial Design and Treatments*

The study was carried out as a randomised complete block design with four replicates per treatment. The plots were located in two adjacent rows and consisted of a trial tree with at least one buffer tree on either side. Trees were selected during full bloom in mid-October 2018, based on uniformity of appearance. One representative branch on the west side of each trial tree, with fruit-bearing wood older than two years, was tagged for treatment application. Within each of the four blocks, trees were randomly allocated to three different treatments: pre-harvest L-proline, post-harvest L-proline, or zero application control.

Unlabelled L-proline ($C_5H_9NO_2$) and isotopically labelled 98 atom-% $^{15}N$-$C_5H_9NO_2$ (both sourced from Sigma-Aldrich Pty. Ltd., St. Louis, MO, USA) were used to prepare a 20 atom-% $^{15}N$-enriched L-proline (L-proline) solution (2000 ppm). To mimic the application by commercial air-assisted spray units, 50 mL of the solution was applied as fine mist from spray bottles onto both the upper and lower leaf surfaces of the selected branches. The same volume of water was used in the control treatment. Pre- and post-harvest treatments were applied in three weekly doses of 100 mg L-proline each, commencing after the last commercial pre-harvest N application at straw phase of fruit development (7 WAFB)

and two weeks after harvest (13 WAFB), respectively (Table 1). Treated branch segments received a total of 300 mg L-proline, i.e., 36.9 mg N, equivalent to 0.5 kg N $ha^{-1}$.

**Table 1.** Phenological stages and key events over the duration of the trial, related to weeks after full bloom (WAFB). Leaf samples were taken at intervals of three weeks, commencing at 7 WAFB and concluding at senescence at 28 WAFB.

| Phenological Stage | Date | WAFB | Key Events |
|---|---|---|---|
| Full bloom | 11 October 2018 | 0 | Tree selection |
| Petal Fall | 25 October 2018 | 2 | |
| Pit hardening | 15 November 2018 | 5 | |
| Straw colour | 29 November 2018 | 7 | Start pre-harvest L-proline; initial leaf samples |
| Full maturity | 28 December 2018 | 11 | Fruit harvest; dissection; quality analysis |
| Early leaf yellowing | 10 January 2019 | 13 | Start post-harvest L-proline |
| Leaf yellowing | 7 February 2019 | 17 | Extension growth removal |
| Leaf senescence | 25 April 2019 | 28 | Last leaf samples |
| Late winter dormancy | 22 August 2019 | 46 | Whole branch harvest and dissection |

### *2.3. Sampling and Measurements*

#### 2.3.1. Leaf Samples

Initial sampling of leaves commenced immediately before pre-harvest application and thereafter at intervals of three weeks until leaf senescence. Samples consisted of 10 mature leaves which were randomly picked from treated branches, washed with distilled water to remove surface $^{15}$N-labelled L-proline, and dried with paper towel. The chlorophyll content (n = 40 per treatment, i.e., 10 leaves × 4 branches) was estimated for each leaf in the first quarter closest to the petiole using a SPAD-502 Plus Chlorophyll Meter (Konica Minolta Sensing, Inc., Osaka, Japan). The 10 leaves were subsequently pooled (n = 4 per treatment) and oven-dried at 60 °C. To investigate the movement of L-proline $^{15}$N following uptake, additional leaf samples were obtained from shoot extension growth at 17 WAFB, immediately before extension shoots were removed as part of standard orchard management practice.

#### 2.3.2. Fruit Samples

Fruit from each treated branch segment was hand-picked at commercial maturity (11 WAFB), counted, and weighed. To determine partitioning of $^{15}$N within the fruit, 10 fruit were washed with distilled water to remove surface $^{15}$N-labelled L-proline and fruit pedicels were detached by hand. Stones were removed with a manual cherry destoner (Westmark GmbH, Lennestadt, Germany) and washed with water to remove any attached flesh. Skin and flesh were carefully separated using a sharp kitchen knife. Fruit tissues (pedicels, stones, flesh, skin; n = 4 per treatment and tissue) were oven-dried at 60 °C. A subsample of 20 blemish-free fruit per branch was obtained for quality assessment, including measurement of weight (electronic balance, Mettler Toledo GmbH, Greifensee, Switzerland), size measured as diameter (DigiMax digital calipers, Wiha Werkzeuge GmbH, Schonach im Schwarzwald, Germany), skin colour (Australian Cherry Colour Guide, Cherry Growers Australia; and Chroma meter CR-400, Konica Minolta Sensing, Inc., Osaka, Japan; with output in the *L*a*b** colour space [19]), compression firmness (FirmTech 2, BioWorks Inc., Wamego, KS, USA), skin puncture force (Güss Manufacturing Ltd., Strand, South Africa), and stem retention force (Series 5 force gauge, Mark-10 Corporation, Copiague, NY, USA) of individual fruit (n = 80 per treatment, i.e., 20 fruit × 4 branches). The 20 fruit were then pooled (n = 4 per treatment) and assessed for total soluble solids (TSS, PR-1 digital refractometer, Atago Co., Ltd., Tokyo, Japan), and titratable acidity (TA, G20 Compact Titrator, Mettler Toledo, Port Melbourne, Australia) according to previously published methods [20]. Total anthocyanin content was determined by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) following methods adapted from Blackhall, et al. [21].

2.3.3. Branch Samples

The branch sections that received treatments were destructively harvested at winter dormancy and dissected into the various plant tissues of buds, spurs, bark, and inner wood (n = 4 per treatment and tissue). All material was oven-dried at 60 °C.

2.3.4. Sample Preparation and Nitrogen Stable Isotope Analysis

The dried leaf, fruit, and branch tissue samples were ground into a fine powder using a ball mill (MM 200, Retsch GmbH, Haan, Germany), in preparation for stable N isotope analysis. The N content (%N) of the samples was determined with an elemental analyser using flash combustion (vario PyroCube, Elementar, Langenselbold, Germany), in which a sample is burned in an excess of oxygen and N converted into dinitrogen ($N_2$) gas for measurement. The $N_2$ gas was then released into a continuous flow mass spectrometer (Isoprime100 with IonVantage software, version 1,7,3,0) to perform the isotopic measurements (Central Science Laboratory, University of Tasmania), according to methods described in Tan, et al. [9].

Analysed $^{15}N$ atom-% ($^{15}N_{apc}$) values were used to calculate the proportion of N within a plant tissue that was derived from L-proline N ($NDP_{tissue}$) by:

$$NDP_{tissue}\ (\%) = \frac{15N_{apc}\ of\ a\ tissue - NA}{N_{Papc} - NA} \times 100;$$

where $N_{Papc}$ represents the $^{15}N$ enrichment of applied L-proline (20 atom-% $^{15}N$) and NA, the natural abundance, as measured from leaf samples taken prior to the application of $^{15}N$-enriched L-proline.

*2.4. Statistical Analysis*

Data were presented as means and standard deviation (SD) for each treatment and were compared using two-sample *t*-tests and one-way analysis of variance (ANOVA) for repeated measures with Tukey's post hoc multiple comparison (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp), with least significant differences calculated at a 95% confidence interval. Sample sizes were n = 4, unless otherwise stated. To evaluate practical significance of the results, effect sizes were determined as Cohen's d (*t*-test) and eta-squared η (ANOVA), respectively. The outcomes of Cohen's d and eta-squared are referred to as small (d = 0.2; η = 0.01), medium (d = 0.5; η = 0.06), and large (d = 0.8; η = 0.14).

## 3. Results

*3.1. Chlorophyll Estimates, Total %N and %NDP of Leaves*

Chlorophyll estimates, total %N and %NDP of leaves are presented in Figure 1.

Leaf chlorophyll estimates were 44.9 ± 2.7 SPAD units at trial commencement, peaking between 10 WAFB and 16 WAFB, and declining at 19 WAFB (Figure 1a). In the pre-harvest treatment, increased chlorophyll levels were recorded after L-proline application at 10 and 13 WAFB, with significant treatment effects at 13 WAFB between pre- and post-harvest treatments ($p$ = 0.008) and close to significant effects between pre-harvest and control treatments ($p$ = 0.066). Following application, there was a significant increase in chlorophyll levels in the post-harvest treatment at 16 WAFB, relative to other treatments. No treatment effects were recorded for the remainder of the trial.

In control trees, total leaf %N was highest at trial commencement (7 WAFB) in late November 2018 at 3.1%, and decreased to 2.0% at leaf senescence in late April 2019 at 28 WAFB (Figure 1b). This trend was mirrored in both the pre- and post-harvest treatments. There were no significant differences in leaf %N between treatments, except for 10 WAFB when %N in the pre-harvest treatment was lower than the control, and 25 WAFB when both pre- and post-harvest treatments had significantly lower %N than the control.

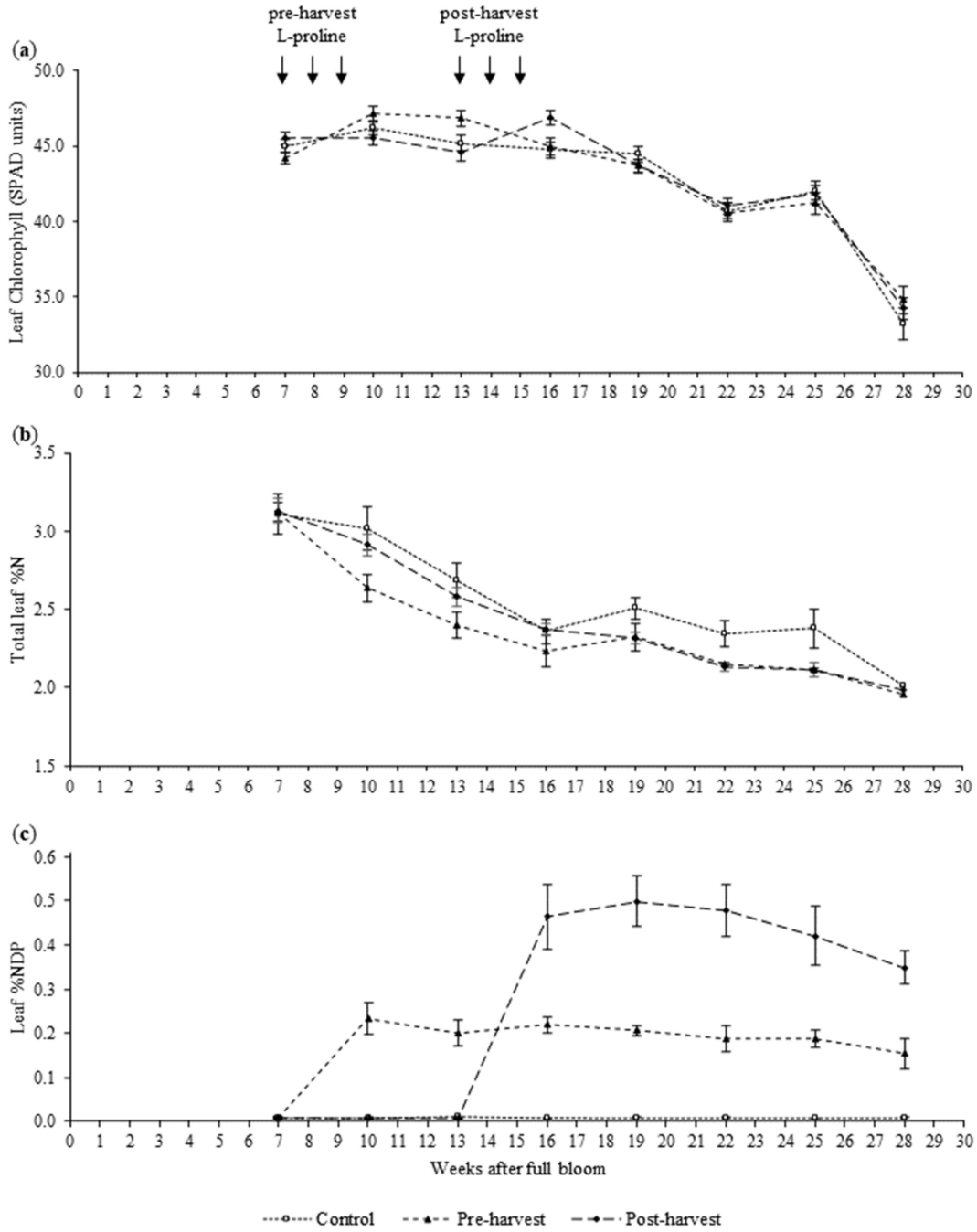


**Figure 1.** (**a**) Chlorophyll estimates (SPAD units; n = 40), (**b**) total leaf N content (%N; n = 4), and (**c**) N derived from L-proline (%NDP; n = 4) from initial sampling prior to the first L-proline application (7 WAFB) until leaf senescence (28 WAFB). Pre- and post-harvest treatments were applied weekly from 7 to 9 WAFB, and 13 to 15 WAFB, respectively. Error bars represent ± standard error of the means (SEM).

Elevated $^{15}N$ levels were measured in leaves that received $^{15}N$-enriched L-proline. After initial uptake, leaf %NDP did not increase as the season progressed for either pre- or post-harvest treatments. Nitrogen derived from L-proline of the pre-harvest application was 0.22% of total N (Figure 1c). Post-harvest uptake of L-proline, resulting in a leaf %NDP of 0.45%, was significantly higher than that of the pre-harvest application. Neither pre- nor post-harvest application of L-proline led to increased $^{15}N$ levels in leaves from new shoot extension growth.

### 3.2. Total %N, %NDP, and Quality of Fruit

Total %N in fruit tissues was in the range of 0.9% and 1.4%, depending on tissue type (Table 2). *t*-tests did not indicate significant differences in %N between fruit tissues from trees that received pre-harvest L-proline and control trees. However, there was a trend of lower %N in the pre-harvest compared with control treatments. Apart from the skin, where Cohen's d indicates a large effect of the treatment on %N, the effect size for all other fruit tissues was in the small to medium range. Measured $^{15}N$ levels were in the range of the natural abundance in the control treatment, whereas fruit of the pre-harvest L-proline application showed elevated $^{15}N$ levels, with the highest %NDP in the pedicel (0.21%), followed by the skin (0.17%), flesh (0.12%), and stone (0.03%; Table 2).

**Table 2.** Total N content (%N) and N derived from L-proline (%NDP) of fruit tissues at commercial harvest arising from trees from control and pre-harvest L-proline application (n = 4).

| | | Control | | Pre-Harvest | | | |
|---|---|---|---|---|---|---|---|
| | | **Mean** | **SD** | **Mean** | **SD** | ***p* Value** | **Cohen's d** |
| Pedicel | %N | 1.06 | 0.15 | 1.08 | 0.05 | 0.853 | −0.14 |
| | %NDP | 0.00 | 0.00 | 0.21 | 0.05 | <0.001 | 5.72 |
| Skin | %N | 1.00 | 0.13 | 0.91 | 0.05 | 0.281 | −0.84 |
| | %NDP | 0.00 | 0.00 | 0.17 | 0.05 | 0.001 | 4.51 |
| Flesh | %N | 1.35 | 0.29 | 1.27 | 0.10 | 0.615 | −0.37 |
| | %NDP | 0.00 | 0.00 | 0.12 | 0.05 | 0.003 | 3.29 |
| Stone | %N | 1.05 | 0.33 | 0.94 | 0.05 | 0.542 | −0.46 |
| | %NDP | 0.00 | 0.00 | 0.03 | 0.01 | 0.002 | 3.81 |

The application of pre-harvest L-proline resulted in significant changes in most fruit quality parameters measured (Table 3). Fruit from trees that received the pre-harvest L-proline treatment showed deeper and darker coloured skin compared with fruit from untreated trees, indicated by significantly higher colour chart scores and lower values for the colour parameters $a^*$ and $b^*$. Fruit weight and size, skin puncture, and stem retention force were significantly lower in the pre-harvest treatment compared with the control. Treatment effects were in the small to medium range, apart from stem retention force, where Cohen's d indicated a large treatment effect, and titratable acidity, with a treatment effect in the medium to large range.

**Table 3.** Selected fruit quality parameters at commercial harvest arising from trees from control and pre-harvest L-proline application.

| | | Control | | Pre-Harvest | | | |
|---|---|---|---|---|---|---|---|
| | **n** | **Mean** | **SD** | **Mean** | **SD** | ***p* Value** | **Cohen's d** |
| Weight (g) | 80 | 12.9 | 1.6 | 12.2 | 1.7 | 0.006 | −0.44 |
| Size (mm) | 80 | 29.7 | 1.4 | 29.1 | 1.6 | 0.007 | −0.43 |
| Colour (chart) | 80 | 4.5 | 0.8 | 4.9 | 0.8 | 0.005 | 0.45 |
| Lightness $L^*$ | 80 | 28.0 | 2.2 | 27.3 | 2.1 | 0.067 | −0.29 |
| Redness $a^*$ | 80 | 27.0 | 5.0 | 24.3 | 5.0 | 0.001 | −0.54 |
| Yellowness $b^*$ | 80 | 7.5 | 2.5 | 6.2 | 2.4 | 0.002 | −0.50 |
| Compression firmness (g mm$^{-2}$) | 80 | 304.2 | 37.0 | 293.7 | 37.4 | 0.076 | −0.28 |
| Skin puncture force (kg) | 80 | 0.39 | 0.04 | 0.37 | 0.05 | 0.040 | −0.33 |
| Stem retention force (g) | 80 | 951.8 | 194.2 | 795.8 | 182.1 | <0.001 | −0.83 |
| Total soluble solids (°Brix) | 4 | 15.4 | 0.4 | 15.5 | 0.8 | 0.835 | 0.15 |
| Titratable acidity (g L$^{-1}$) | 4 | 6.0 | 0.4 | 5.7 | 0.1 | 0.372 | −0.68 |
| Total anthocyanins (mg 100 g$^{-1}$) | 4 | 15.7 | 6.0 | 18.4 | 4.2 | 0.492 | 0.52 |

### *3.3. Total %N and %NDP of Branches*

Total %N of winter-harvested and dissected branches was highest in the buds (1.9%), followed by spurs (1.2%), bark (1.0%), and inner wood (0.3%), but for each tissue there were no significant differences between treatments (Figure 2a). All examined tissues of the branches that received L-proline showed elevated $^{15}$N levels, resulting in significantly higher %NDP than the control (Figure 2b), with the highest %NDP determined in the buds, spurs, and bark. In branches that received post-harvest L-proline, %NDP was significantly higher in the buds, wood, and bark, compared to the pre-harvest treatment, with η treatment effects in the large range.

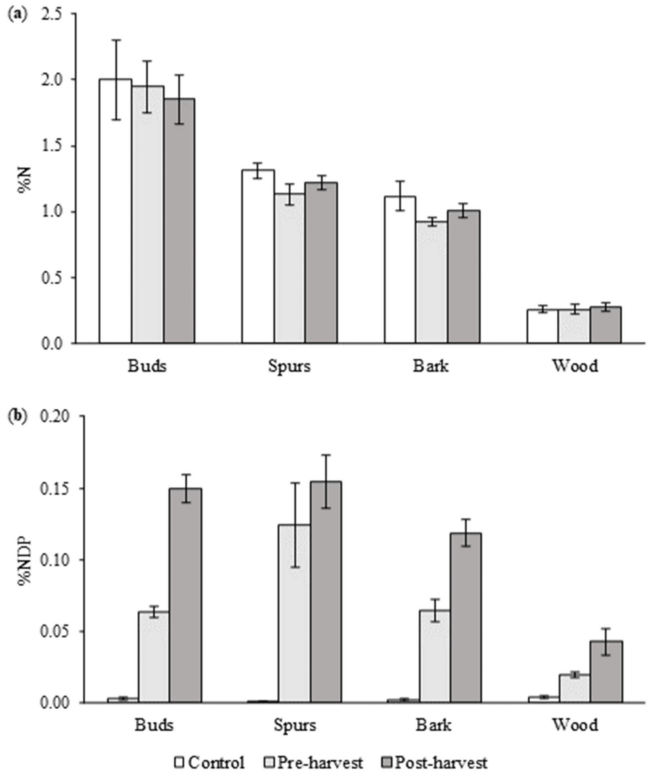


**Figure 2.** (**a**) Total N content (%N) and (**b**) N derived from L-proline (%NDP) of branch tissues at dormancy. Error bars represent ± SEM (n = 4).

## 4. Discussion

This study demonstrates that the amino acid L-proline was taken up by mature sweet cherry leaves. Both pre- and post-harvest application resulted in the recovery of L-proline in leaves, fruit, and branches, with the %NDP being affected by application timing. Pre-harvest foliar application of L-proline was associated with darker fruit with lower stem retention, indicating advanced maturity under this treatment.

The lack of a significant increase of %N in the leaves, fruit, and branches, following the application of L-proline, was anticipated due to the nature of the experiment as a proof-of-concept study where a low rate of the $^{15}$N-labelled fertiliser was applied. Overall, the percentage of N derived from L-proline in tree tissues was low. Due to the deciduous nature of sweet cherry, remobilised N from previous seasons and N from commercial fertiliser application (80 kg N ha$^{-1}$ and season) likely accounted for the majority of N present in tissues. L-proline application was a relatively minor contribution of 0.5 kg N ha$^{-1}$ to the overall N fertiliser budget.

### *4.1. Leaves*

The general decline of total leaf %N throughout the season until senescence was consistent with a trend observed in deciduous fruit trees [9]. The highest %N at 7 WAFB co-

inciding with the last commercial N application, and a steady decline afterwards, indicated the typical translocation of N from leaves to other organs, e.g., the fruit. After fruit harvest, the decline in %N continued, coinciding with the commencement of leaf senescence, and withdrawal of the leaf N to other organs [9,22]. The general pattern of leaf %N decline after harvest is mirrored in the pattern of estimated chlorophyll, which suggests a relationship between leaf N status and SPAD meter readings [23].

In mature leaves, elevated $^{15}$N levels were detected at the first sampling date after application (Figure 1c). Lower %NDP in leaves that received pre-harvest L-proline coincided with elevated $^{15}$N levels in fruit tissues, indicating that a portion of the L-proline was translocated to fruit (Table 2) as a competitive sink for nutrients. It is probable that due to the nature of the foliar application aimed at mimicking standard orchard practice, some L-proline may have been directly taken up by other parts of the branch, including the fruit. Other contributing factors to the lower %NDP may be of external nature, such as specific weather conditions throughout the pre-harvest period, with more frequent rain events potentially leading to wash-off and/or altered relative humidity, affecting uptake and incorporation into the leaf structure [11].

Independent of the timing of application, the %NDP in treated mature leaves remained stable throughout the season with a declining trend towards leaf senescence, when %N was at its lowest. This is in agreement with Thielemann, et al. [24], who reported a decline of $^{15}$N content of urea-treated cherry leaves which coincided with an increase of $^{15}$N detected in other organs such as the buds, bark, and wood, and was attributed to translocation of $^{15}$N to storage organs later in the season. It could be inferred that the translocation to other organs within or out of the treated branch was similarly occurring in the cherry trees of this study. The significantly higher leaf %NDP effects for post-harvest application could have implications for higher N available for withdrawal and translocation prior to senescence, and provide additional internal reserves for the subsequent season.

The lack of increase in $^{15}$N levels in leaves of extension growth suggests that L-proline was not translocated to these tissues in detectable amounts during the growth phase of the tree. This indicates that L-proline is assimilated where applied, before its translocation to alternative sinks along with other stored N, i.e., to developing fruit at pre-harvest application and/or to storage organs both pre- and post-harvest. It is likely that the N requirements of extension growth were met by soil uptake via the xylem stream [9], and therefore that the sink strength of new shoots for translocated N from L-proline was not as strong as that of other organs.

It appears that L-proline application had a physiological impact on mature leaves, as suggested by significantly elevated leaf chlorophyll levels soon after application of both the pre- and post-harvest treatments, i.e., at 13 and 16 WAFB, respectively. At this time, no additional N was applied as part of commercial orchard management practice, hence the only difference between the treatments was L-proline application. El Sayed, et al. [17] reported increased chlorophyll content in leaves of pomegranate after application of L-proline, whereas Takeuchi, et al. [15] were only able to detect increased chlorophyll levels with L-proline application when initial chlorophyll levels were low and soil N was not in excess. When initial chlorophyll levels were already high, the application of L-proline did not affect chlorophyll content [15]. In our study, the elevation in chlorophyll levels after L-proline application was of a temporary nature, and further research is needed to explore any potential physiological significance in sweet cherry.

### *4.2. Fruit*

L-proline application did not significantly alter fruit N status, which was likely attained by remobilised N from previous seasons, native soil N supply, and pre-harvest fertiliser application [6,7]. Interestingly, evaluation of Cohen's d revealed a large effect in skin %N, which was lower in fruit of branches treated with pre-harvest L-proline (Table 2), while effects on the flesh and stone were in the small to medium range. It is difficult to reconcile

these findings and it remains to be investigated whether this phenomenon can be replicated in larger-scale studies and, if so, what the underlying mechanism may be.

The %NDP after pre-harvest L-proline application was highest in pedicels, followed by the skin and flesh. The stone showed a significantly lower %NDP, potentially due to the timing of the application being relatively late in fruit development at straw phase, when the stone was already developed. The overall increased $^{15}$N levels in fruit tissue are indicative of direct assimilation and/or translocation of L-proline into sweet cherry fruit tissue following pre-harvest application commencing at straw phase of fruit development.

In this study, significant differences in most fruit quality parameters were observed. Fruit from trees that received pre-harvest L-proline were smaller, yet deeper in colour, and showed reduced skin puncture and stem retention force. These significant treatment effects in combination with reduced titratable acidity, although not statistically significant, indicate that L-proline application may enhance fruit maturity. This may have implications on the timing of commercial harvest, which is commonly determined by skin colour assessment, and revenues, which are generally higher for large and firm fruit with a high stem retention. Although statistically significant, the differences in fruit quality parameters are small and may not lead to commercial consequences.

Although %N of fruit tissues did not significantly differ between control and pre-harvest treatment, the observed trend towards lower %N in fruit from trees that received L-proline may partially explain some of the fruit quality outcomes. Colour development in sweet cherry is dependent on the biosynthesis and accumulation of anthocyanins. Although not significant due to a small sample size (n = 4), fruit from the pre-harvest treatment showed higher anthocyanin contents compared with the control, coinciding with significantly higher skin colour measurements. Research suggests that anthocyanin biosynthesis, and subsequent colour development, is enhanced under low N conditions [25,26]. This relationship was also observed in sweet cherry in a study evaluating the effect of different rates of soil-applied N on fruit quality, where reduced N application resulted in enhanced skin colour (Hölzel, et al. unpublished data).

From the design of the experiment, it is not possible to determine a cause-effect relationship between the application of pre-harvest L-proline, N status, and fruit quality outcomes. Although differences in fruit quality parameters revealed a trend towards enhanced maturity of fruit treated with pre-harvest L-proline, this trend needs to be interpreted cautiously due to the small sample sizes (one treated branch per tree) and the general lack of information regarding the ideal timing and rates of L-proline application for sweet cherry. To confirm this trend, studies with whole-tree or whole-block replicates and higher number of treatments (timing, doses) are necessary to evaluate L-proline application as a possible tool for short-term maturity and quality management in cherry orchards.

### *4.3. Branch*

Overall N status in branch tissues was not influenced by the application of L-proline, suggesting that internal storage, native soil N supply, and commercial N application were the main contributors to %N in branches.

Elevated $^{15}$N levels in all branch tissues at late dormancy indicate a translocation of $^{15}$N from leaves to storage tissues, as reflected in the pattern of leaf %N decline demonstrated in this study. The %NDP varied depending on the branch tissue and L-proline application timing. The lower %NDP for all branch tissues in the pre-harvest treatment was mirrored in leaf %NDP and can be attributed to the fruit being a strong sink for pre-harvest L-proline, whereas this competing sink was absent in the post-harvest treatment. Higher %N and higher %NDP in the buds, spurs, and bark, compared with wood, indicate that these tissues had a higher sink activity for N. Ayala, et al. [12] reported a similar enhanced sink strength for bark compared with wood after application of $^{15}$N-urea to branches of 'Bing' on 'GI®6'. In our study, destructive harvest of branches occurred at a late stage of dormancy, immediately prior to bud burst. High %N and %NDP in buds and spurs may be attributed to the potential remobilisation of N reserves for new season growth. With reference to

Thielemann, et al. [24], who recovered $^{15}N$ in young leaves and fruit in the season following urea application, it is likely that stored $^{15}N$ derived from L-proline is also available for new season growth. The significantly higher uptake and recovery of $^{15}N$ from L-proline from post-harvest application in wood, buds, and bark indicates a strong possibility of subsequent season effects due to the deciduous nature of sweet cherry. However, this needs to be confirmed by further research.

## 5. Conclusions

This study demonstrates the uptake of foliar-applied L-proline into mature leaves of sweet cherry and its translocation into fruit and storage tissues of the branch. The fruit was a sink for L-proline applied pre-harvest, with quality outcomes indicating a trend towards enhanced maturity. Branch tissues were a sink for both pre- and post-harvest L-proline, with potential remobilisation of N for the coming growing season. This proof-of-concept study sets the foundation for large scale trials, using higher rates and optimised timing of L-proline application, to achieve desired fruit quality and N storage outcomes in sweet cherry.

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**Data Availability Statement:** Data supporting this manuscript is not publicly available but can be made available upon reasonable written request to the corresponding author (D.C.C.).

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