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Plant Biostimulants Enhance Bud Break in *Vitis vinifera* Crimson Seedless Using Combination Treatments

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Abstract: The rest-breaking agent, hydrogen cyanamide (HC), can substitute insufficient chill unit accumulation in *Vitis vinifera* and induce uniform bud-break; however, due to its toxicity it is being banned. In South Africa, red seedless grapes, including *V. vinifera* Crimson Seedless (CS), are the largest table grape export group; therefore, replacing HC in *V. vinifera* CS is crucial. This study aimed to confirm the molecular triggers induced by HC and assess the bud-break-enhancing abilities of commercial plant biostimulants. Forced bud-break assay experiments using *V. vinifera* CS single-node cuttings and a small-scale field trial were performed. Results demonstrated that increased chill unit accumulation (CUA) reduced HC efficacy. Bud-break started between 10 and 20 days after treatment, irrespective of final CUA. The small-scale field trial found that HC 3% and biostimulants were similar to the negative control. The treatment of dormant grapevine compound buds with nitric oxide (NO), hydrogen peroxide (H_2O_2), and hypoxia trigger dormancy release to a certain extent, supporting the molecular models proposed for HC action. NO, H_2O_2, and hypoxia, in combination with PBs, may potentially replace HC; however, this needs to be confirmed in future experiments.

Keywords: *Vitis vinifera*; Crimson Seedless; hydrogen cyanamide; bud break; chill unit; single-node cuttings; plant biostimulant; nitric oxide; hydrogen peroxide; hypoxia

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1. Introduction

Woody perennial crops survive cold winter months by suspending visible growth. This phenomenon is called dormancy [1]. When crops are dormant, compound buds are covered by bud scales and their vascular systems are physically separated from the rest of the vine [2]. This protects reproductive tissues, which were formed during the previous growth season. Only upon environmental cues (e.g., warming and increasing daylight hours) do the vascular systems of the buds re-connect to the cane for growth and differentiation to begin [2]. This is known as bud-break, defined as the day the first green tissue becomes visible underneath the bud scales [1]. Rapid, uniform bud-break is vital for optimal fruit set and quality, which is directly affected by the number of chill units (CU) accumulated during endodormancy.

Vitis vinifera generally requires a minimum of 200 h of exposure to temperatures between 0 and 10 °C to achieve a high bud-break percentage, defined as a bud-break of 80% or above [3]. Furthermore, to achieve high and uniform bud-break percentages across vineyards, a minimum exposure time of 400 h at 3 °C is necessary [3]. However, slight variations in cold accumulation requirements may occur due to crop and cultivar differences [4]. CU models provide climate predictions and trends that affect the development of grapevine compound buds and can thus be used to determine optimal farming strategies.

Commonly used CU models include the Dokoozlian model, the Richardson (Utah) model, the Infruitec model (daily positive Utah model), and the dynamic model [4–8]. The Dokoozlian model calculates the ratio between exposure to cold temperatures (hours < 7 °C) and cold-negating temperatures (hours > 20 °C) [4]. The Richardson and Infruitec models are very similar regarding unit accumulation by allocating units per hour and suggesting that full units are accumulated at temperatures between 2.5 and 9.1 °C [8]. Earlier work by Fuchigami et al. looked at developing a degree growth stage model for woody plant species [9]. The dynamic model is more complex, taking multiple factors into account, including sequences of both cold and hot temperatures during winter, and determines how much chill was reversed by the latter [6–8]. Furthermore, it is more accurate than the other models [8,10]. Nevertheless, all these models are used by South African fruit growers [4,8]. The transition of endodormancy to ecodormany is a critical phase needed to be accounted for when applying treatments such as HC [1,9]. Trejo-Martínez et al. [11] followed the changes in metabolic heat production along dormancy and dormancy release. Such approach has been successfully used in apples, and export pecan nuts and table grapes in the mild winter in north-west Mexico [11]. The choice of which model to use depends on the preferences of individual farmers and field experts.

South Africa has five table grape production regions: the Northern Provinces, Orange River, Olifants River, Berg River and Hex River (SATI 2018). The Northern Provinces, Orange River and Olifants River accumulate insufficient CU to achieve high percentages of bud-break [4,12]. Whilst the Berg River and Hex River regions accumulate enough CU for a high percentage bud-break, they do not always accumulate enough CU for uniform bud-break. The rest-breaking agent Dormex® (Alzchem Trostberg GMBH, Trostberg, Germany) of which the active ingredient is 49% (*w*/*v*) hydrogen cyanamide (HC), can substitute insufficient chill unit accumulation (CUA) in crops and induce uniform bud-break. Dormex® is typically applied at a concentration of 3% or 5% (*v*/*v*) about four weeks before the expected onset of natural bud-break.

Regardless of cold accumulation in South Africa, farmers apply HC to guarantee high bud-break percentages and rapid and uniform dormancy release in their vineyards [4]. The need for the use of HC by fruit growers is predicted to increase with the effects of climate change and a general warming of growing regions [13]. HC is toxic, and its use has either already been banned or is predicted to be banned in several countries [14]. This is especially concerning as markets are sensitive, emphasizing the importance of looking for environmentally friendly production practices for grape products. The five largest export markets for South African table grapes are the European Union (54%), United Kingdom (22%), Canada (6%), Middle East (5%), and Southeast Asia (5%) [15]. Within each of these markets, excluding Southeast Asia, red seedless grapes are the largest table grape export group, including *V. vinifera* Crimson Seedless (CS) [15]. *V. vinifera* CS is a late-season red seedless table grape cultivar and is the most widely planted table grape cultivar in South Africa, with 3798 and 3660 hectares planted in 2021 and 2022, respectively [15]. Therefore, finding a replacement for HC in *V. vinifera* CS should be a priority for the South African agricultural sector.

A significant amount of research to date has focused on the molecular mode of action of HC on grapevine compound buds [1,16–25]. Recently, two models of the molecular mode of action of HC and bud-break have been reported [26,27]. Briefly, HC inhibits catalase (CAT), which creates a hypoxic environment in the buds, subsequently inducing fermentative metabolism, which is associated with an increase in nitric oxide (NO) and reactive oxygen species (ROS) [28]. The subsequent ROS and NO species signalling triggers the expression of transcription factors that upregulate genes related to growth and differentiation [26,27]. Additionally, phytohormone profiles change in the buds during dormancy release. For instance, abscisic acid (ABA) is well known to maintain bud dormancy, whilst ethylene is associated with dormancy release [27]. Furthermore, in grapevine buds, the exogenous application of ethylene has both been found to enhance dormancy release and act as a key signalling molecule within the antioxidant defence system [19,24].

The proposed molecular mechanisms of HC have assisted in developing less toxic commercial alternatives, such as plant biostimulants (PBs), which have been sold amongst agrochemical products over the last few years. Indeed, several PBs have shown a potential to enhance bud-break, compared to no treatment, in apple, grapevine, pear, sweet cherry, blackberry, peach, and kiwi [29–36]. However, very few reports, especially in the grapevine, exist of PBs that enhanced bud-break in a similar or superior manner to that of HC. Nevertheless, the success of PBs reported in bud-break studies may be attributed to their ability to cause major oxidative stress in plants whilst providing nutrients necessary for cellular and tissue recovery [37]. It is typical for such treatments to be amino acid- and nitrite salt-based and to be applied in combination with an adjuvant or oil-based substance as well as the additional supplementation of NO and calcium-containing biochemical agents [37]. Interestingly, all these elements have been reported in the molecular models of HC action during bud-break [26–28].

Oxidative stress induced by PB-based treatments is likely attributed to the formation of respiratory stress and NO formation. Nitrites and nitrates form enzymatically via the activation of nitrogen nitrite and nitrate reductases under hypoxic conditions [32]. These conditions can be artificially created by commercial rest-breaking oils or oil-based adjuvants. Respiratory stress induced by rest-breaking oils leads to ROS accumulation, such as hydrogen peroxide (H_2O_2), a molecular trigger for various processes involved in dormancy release [28]. Calcium is an essential nutrient for plant growth, which enters plant cells in its divalent cation form, calcium ion (Ca^{2+}), but can be toxic when present in excessive amounts [38]. ROS, such as H_2O_2, produced by NADPH oxidase, activate Ca^{2+} channels, which causes cell expansion and growth [39].

NO is a type of ROS used and produced by plants and has several important regulatory functions. These include abiotic and biotic stress responses and are involved in various physiological processes, including dormancy release [40]. NO is a well-known trigger of dormancy release in seeds and bulbs [41,42]. Additionally, its accumulation in grapevine buds has also been well-documented after the exogenous application of HC [26,27,32]. Indeed, NO can inhibit cytochrome oxidase by competing with oxygen and reversibly inhibiting CAT [43,44]. Whilst the success of dormancy-breaking agrochemicals is attributed to their ability to induce oxidative stress, uncontrolled increases in ROS accumulation can damage cellular components [45].

Agrochemicals with high amino acid contents may protect against uncontrolled ROS levels. Indeed, changes in amino acid profile are associated with plants under major oxidative stress, and the exogenous application of several amino acids is known to improve antioxidant defence responses [46]. Protein degradation, which occurs because of oxidative stress, releases amino acids, which the plant uses to synthesise various protective metabolites. For instance, proline, arginine, glutamine, asparagine, and gamma-aminobutyric acid (GABA) are used to produce osmolytes or secondary metabolites, or are stored as organic nitrogen. Interestingly, GABA has been found to reduce H_2O_2 during grapevine bud-break by activating *VvCAT2* while repressing *VvCAT1*, both of which are isogenes of *CAT* [24]. Furthermore, aromatic amino acids, such as phenylalanine and tyrosine, are direct precursors for pathways related to hormone, polyphenol, and cell wall biosynthesis [46]. Similarly, branch-chain amino acids, such as isoleucine and valine, are used to produce cyanogenic glycosides, glucosinolates and acyl sugars. Finally, sulphur-containing amino acids, such as methionine and cysteine, play key roles in sulphur metabolism, which shifts during oxidative stress. This certainly emphasizes the importance of including amino acids as a key ingredient in treatments which induce bud-break.

A common method for studying dormancy in grapevine is single node cuttings (SNCs) under forced conditions. This approach is useful, as buds display behavioural similarities to those in the vineyard [19,24,47]. Another benefit of such an approach is that it enables the evaluation of the depth of dormancy via eliminating para- and ecodormant factors. Considering this, the current study aimed first to confirm the various molecular triggers induced by HC in dormant grapevine buds [26,27]. This was achieved via the external

application of mineral oil, H_2O_2, and ethylene and two slow-releasing NO donors: diethylamine NONOate sodium salt hydrate (NONOate) and S-nitroso glutathione. The second aim was to assess the bud-break-enhancing abilities of four commercially available PBs: a riboflavin derivative (7,8 dimethylalloxazine), a citrus-based plant extract, an L-glutamic acid-based agrochemical, and a nitrogen and amino acid-based agrochemical. These aims were achieved via the execution of a series of forced bud-break assay experiments using *V. vinifera* CS SNCs over two years (with material from two growth seasons) and performing a small-scale field trial, which was conducted in the second year.

2. Materials and Methods

2.1. Plant Material

All assays were conducted using *V. vinifera* CS plant material from the 9-year-old CS block (Ramsey rootstock) at Windmeul Farm, Hoekstra Fruit, in Paarl, Western Cape, South Africa. Each row in the CS vineyard block consists of twenty-one line posts, with four vines between posts. The open gable trellis design consists of six catch wires, of which the bottom three are typically used for binding the one-year-old canes after winter pruning (Figure 1a).

Figure 1. Photos taken during glasshouse forced bud-break assays of *V. vinifera* Crimson Seedless (CS) plant material: (**a**) Gable trellis system of CS vineyard block on Windmeul Farm, Hoekstra Fruit, Paarl. (**b**) Single-node cuttings (SNCs) placed upright in trays filled with tap water prior to treatment applications. (**c**) SNCs that are positioned in styrofoam supports and floated on water in plastic trays at 22 °C, with a 16/8 h light/dark photoperiod. (**d**) Green tissue and leaf tips visible underneath the bud scales, specifically E-L stage 3, and E-L stage 4 (arrow pointing towards the greening bud) [1,48].

2.2. Chill Unit Recordings

Local weather data were acquired from the Stratus 200 4G weather station (Hortec, Somerset West, Western Cape, South Africa, https://www.hortec.co.za/) (accessed on 22 September 2022, which is located on Môrewag farm, Paarl (approx. 3.6 km from the experimental site). The CUs in this study were expressed as Infruitec Units (IUs) as this is the measure commonly used by local farmers and industry experts [8].

2.3. Forced Bud-Break Assays

2.3.1. Preparation of Plant Material, Experimental Design and Data Collection

Dormant ten-node canes in E-L stage 1 [48] were collected from three middle rows of the CS block during May, June and July (2021 and 2022), before the accumulation of the final total CU required for each experiment (Figure 1a). Canes were separated into SNCs. These SNCs were soaked in contact fungicide (Captab 500 g/L), drained for a few minutes, and placed into heavy-duty plastic bags, then sealed.

The SNCs were stored at 4 °C in darkness until the pre-determined total number of IUs was reached. Finally, the exposed buds were dipped in the various respective treatments. To prevent dehydration of the SNCs in between treatment steps, such as the separation of canes into SNCs and treatment application, they were placed upright in trays filled with tap water (Figure 1b). Following treatment, SNCs were positioned in styrofoam supports and floated on water in plastic trays at a bud-break forcing temperature of 22 °C, with a 16/8 h light/dark photoperiod and light intensity of 35 µmol/m^2/s (F36W/GRO, GRO-LUX T8, Sylvania, Germany, https://www.sylvania-lighting.com/) (accessed on 10 September 2022), (Figure 1c) [4]. Temperature was monitored with Tinytag TGP-4500 loggers (RS Components, Midrand, South Africa, https://za.rs-online.com/web/) (accessed on 10 September 2022), (Figure S1 in Supplementary Material). The location of individual SNCs in the supports was determined using randomized block experimental designs. The water in the trays was replaced every 14 days.

All laboratory equipment used for treatment preparation was thoroughly rinsed with distilled water (dH_2O) before use. The SNCs were monitored for bud-break daily, with the bud-break date being defined as the date at which green tissue became visible underneath the bud scales (Figure 1d) [1]. More specifically, this is defined as E-L stage 3 and E-L stage 4 [48].

2.3.2. Candidate Treatments and Application Concentrations

During the 2021 and 2022 winter seasons, a series of forced bud-break assays were performed, which varied in the final accumulated CU. In each experiment, dH_2O served as the negative control and 3% Dormex® (Alzchem Trostberg GMBH, Trostberg, Germany) as the positive control (referred to as dH_2O and HC 3%, respectively, in all experiments). In 2021, several biochemical agents and two PBs (BC204 and lumichrome) were tested individually (Table 1). In 2022, two agrochemical-based PBs were tested at the manufacturers' recommended concentrations and lower concentrations, along with a mixture treatment of all promising biochemical agents and PBs included in the 2021 assays (Table 2). At the request of the supply companies, all commercial biostimulant and bud-break product treatments were assigned code names.

Table 1. Biochemical agents and plant biostimulants (PBs) tested on *V. vinifera* CS grapevine SNCs in 2021 during forced bud-break assays. Each treatment was assigned a code name.

Treatment	Relevance to Dormancy-Release Molecular Models	Application Concentration	Treatment Code Name
Dormex®	Positive control	3% *v*/*v* [49]	HC 3%
Distilled water	Negative control	NA	dH_2O
H_2O_2	ROS	1% *v*/*v* [24]	H_2O_2 1%
S-Nitrosoglutathione	NO donor	10 µM [50]	SNO 10 µM
Diethylamine NONOate sodium salt hydrate	NO donor	10 µM [50]	NONOate 10 µM
Lumichrome	Riboflavin derivative	5 nM [51]	Lum 5 nM
BC204 (Commercial PB)	Citrus-based plant extract	0.05% *v*/*v* [52]	BC204 0.05%
BUDBREAK® mineral oil	Hypoxia	3% *v*/*v* [53]	BB 3%
Ethephon	Ethylene supplement	0.206% *v*/*v* [24]	

Table 2. Biochemical agents and PBs tested on *V. vinifera* CS grapevine SNCs in 2022 during forced bud-break assays. Each treatment was assigned a code name.

Treatment	Relevance to Dormancy-Release Molecular Models	Application Concentration	Treatment Code Name
Dormex®	Positive control	3% *v*/*v*	HC 3%
Distilled water	Negative control	NA	dH_2O
SN (Commercial PB)	Amino acids formulations (SBB-01), synergistic effect provided by a calcium supplement (NDY-01)	2% *v*/*v* SBB-01 and 20% *v*/*v* NDY-01 (recommended by manufacturer)	SNH
SN		0.2% *v*/*v* SBB-01 and 2% *v*/*v* NDY-01	SNL
PBX (Commercial PB)	Amino acid formulations which alter metabolism in plants	1.5% *v*/*v* (recommended by manufacturer)	PBXH
PBX		0.15% *v*/*v*	PBXL
Combination of selected biochemical agents (refer to Table 1)	Refer to Table 1	1% *v*/*v* hydrogen peroxide, 10 µM S-nitrosoglutathione, 10 µM diethylamine NONOate sodium salt hydrate, 5 nM lumichrome, 0.05% *v*/*v* BC204 and 3% *v*/*v* BUDBREAK® mineral oil	MIX2

2.4. Small-Scale Field Trial Dormancy Release Assay

2.4.1. Experimental Site and Design

A small-scale field trial was conducted to test the two PBs included in the glasshouse assays of 2022. This trial was executed during September and October 2022 in two middle rows of the CS block. Treatments were applied in replicates of eight groups of two plants between individual sets of line posts, using a randomised block design. The two middle vines within each post were the experimental targets. Four canes facing opposite directions of the vine, bound to the bottom wire of the gable trellis system, were selected as experimental target canes. The first 10 buds of each cane were clearly marked for analysis using white acrylic paint, starting from the head of the vine (Figure 2A). To prevent contamination by commercial spray applications, three guard rows on either side and at least two guard line posts on the terminal ends of each row were included in the experimental design (Figure 2B).

B.

Line post number

Treatment replicates / Row	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Guard																					
Guard																					
Guard																					
Target 1			dH_2O	PBXH	HC 3%	dH_2O	PBXH	HC 3%	PBXH	dH_2O	SNH	HC 3%	PBXH	SNH	HC 3%	SNH	PBXH	dH_2O			
Target 2			SNH	HC 3%	PBXH	SNH	SNH	dH_2O	HC 3%	SNH	dH_2O	PBXH	HC 3%	dH_2O	dH_2O	PBXH	SNH	HC 3%			
Guard																					
Guard																					
Guard																					

Figure 2. Small-scale field trial experimental design: (**A**) *V. vinifera* CS target cane for small-scale field trial, bound to the bottom wire of the gable trellis system. Ten target compound buds were clearly

marked for analysis using white acrylic paint, starting from the head of the vine. (**B**) Experimental rows of *V. vinifera* CS block of Windmeul farm, Paarl. Two plant biostimulants, SNH and PBXH, were evaluated against 3% Dormex® (HC 3%), the positive control, and dH_2O, the negative control in the months of September and October 2022. One biological repeat of each replicate consists of the two middle vines of the four planted between line posts. Four target canes were selected per target vine, of which the first ten target compound buds from the base of the vine were marked.

2.4.2. Treatments Evaluated and Dosage Concentrations

Treatments evaluated during the small-scale field trial include SNH and PBXH (Table 2). The positive control was 3% Dormex®, while the negative control was the tap water used to make up the treatments on the farm (which was referred to as dH_2O regardless). With the assistance of Curativo (Pty) Ltd (Wellington, South Africa), treatments were applied at a spray volume of 611 L/ha using STHIL SR 430 backpack mist blowers (Stuttgart, Germany, https://www.stihl.com/default.aspx) (accessed on 1 July 2022) within the first three days after winter pruning had taken place (approx. two weeks before expected natural bud-break date). Target compound buds were monitored daily for dormancy release, using E-L stage 4 as a reference [48].

2.5. Data Handling

Cumulative bud-break was calculated for respective treatments over time. Each assay was analysed as a separate experiment, regardless of the final cumulative CU. Specifically, the bud-break percentage was calculated for each day after treatment application (Equation (1)) [4,32].

$$Bud\ break\ \% = \frac{number\ of\ compound\ buds\ released\ from\ dormancy}{total\ number\ of\ SNCs\ in\ experiment} \times 100 \quad (1)$$

2.6. Statistical Analysis

Using the R package drc, a four-parameter logistic growth curve was fitted to determine the final bud-break percentage, rate, and half maximal effective concentration (EC_{50}) (Equation (2)) [54], and a piecewise linear regression model was fitted to determine the onset (Equation (3)) [32].

$$y = lower + \frac{upper - lower}{1 + e^{slope \times (x - EC50)}} \quad (2)$$

$$y = y_1 \times (x < x_1) + y_2 \times (x > 2 \times EC50 - x_1) + \frac{y_1 + (y_2 - y_1) \times (x - x_1)}{2 \times (EC50 - x_1)} \times (x \geq x_1) \times (x \leq 2 \times EC_{50} - x_1) \quad (3)$$

Listed below are the four parameters which were used to assess bud-break datasets of the different treatments, along with their definitions according to Equations (2) and (3) (Figure S2):

- Onset: The number of days until the bud-break date was reached. Larger positive values indicate delayed time until onset was reached.
- Bud-break rate: The slope between 0% bud-break and final bud-break % indicates bud-break rate between the first and last buds that have broken. The slope parameter (or more generally termed "hillslope") indicates the steepness of decrease. Thus, the larger the slope value, the steeper the decrease. For an increasing function (such as in the current study), the opposite is true: the larger the negative slope is, the steeper the increase, and the faster the bud-break rate.
- EC_{50}: Number of days to reach 50% bud-break after treatment. This is also a parameter indicating speed. Larger positive values indicate delayed time until EC_{50} was reached.

- Final bud-break percentage: The upper value of the cumulative bud-break percentage growth curve (plateau). Larger positive values indicate a higher final bud-break percentage.
- An unadjusted post hoc t-test, with a 5% confidence interval ($p < 0.05$), was used to determine significant differences between growth curve parameters [55].

3. Results

3.1. Chill Units in the Vineyard

CU recordings, which were measured as IUs in the vineyard by the Môrewag Stratus weather station, were used for all dormancy-release assays in the current study, including glasshouse forced bud-break trials performed in the laboratory and the small-scale field trial (Figure S3). In 2021, 57.5 CUs were accumulated by the end of May and reached a maximum of 795.5 CU by the end of October. In 2022, a total of 63.5 CUs was accumulated by the end of May, and maximum of 662 CUs were reached by the end of October. Furthermore, during each month after May, a higher final monthly number of CUs were accumulated in 2021 than in 2022. In both years, CUs started to accumulate in May, reaching final values of 57.5 (2021) and 63.5 (2022), respectively. By the end of July in both years, enough CUs (479.5 in 2021, and 368 in 2022) had accumulated for rapid, uniform dormancy release to take place in the *V. vinifera* CS block [3,56,57].

3.2. Forced Bud-Break Assays

3.2.1. Evaluation of Plant Biostimulants (BC204 and Lumichrome), and Individual Biochemical Agents

During June 2021, the effects of the following treatments were evaluated on bud-break in *V. vinifera* CS SNCs after 100, 200 and 400 CUA: HC 3%, dH_2O, BC204 0.05%, Lum 5 nM, H_2O_2 1%, Eth 1000 ppm, BB 3%, SNO 10 µM, and NONOate 10 µM (Table 1). Each treatment consisted of 30 biological replicates. All samples were collected on 31 May 2021, prepared, and left at 4 °C until the desired final total CU had accumulated. At the time of sampling, 57.5 natural CU had already accumulated in the vineyard (Figure S3).

Cumulative plots of bud-break % across the three experiments showed that with increased CUA, the effect of Dormex® (HC 3%) was reduced in efficacy compared to both the negative control and other treatments (Figure 3). This indicates that there was less need for Dormex® with increased CUA. An interesting observation was that the days until bud-break was first observed lengthened with increasing CU values. After 100, 200 and 400 CUA, bud-break was first observed after about 9 (Figure 3A), 16 (Figure 3B) and 22 (Figure 3C) days, respectively. These longer periods before bud-break onset with more CUA may be due to the increased exposure to cold stress of SNCs, which had accumulated more CUs during storage for artificial cold accumulation. Nevertheless, clear trends were observed amongst the experiments after careful evaluation of bud-break onset, rate, EC_{50} and final cumulative % datasets. It is possible the buds were in a more ecodormant then endodormant metabolic state, which could explain these data.

After 100 and 200 CUA, HC 3%-treated buds took 9.86 and 16.12 days to reach onset, respectively, significantly less than the other treatments ($p = 0$) (Table 3). In contrast, after 400 CUA, HC 3%-treated buds had an onset of 25.4 days, similar to the other treatments. The negative control, dH_2O, yielded onset values of 20.07, 22.17 and 27.08 days after 100, 200 and 400 CUA, respectively. Two treatments, H_2O_2 1% and BB 3%, resulted in significantly less days for bud-break onset to be reached than dH_2O in all three experiments ($p = 0$). Furthermore, the onset after BB 3% treatment was earlier than that of H_2O_2 1% in each experiment. The onset of H_2O_2 1%-treated SNCs was 18.8, 21.07 and 24.48 days; for BB 3%, they were 16.48, 19.16 and 25.17 days after 100, 200 and 400 CUA, respectively.

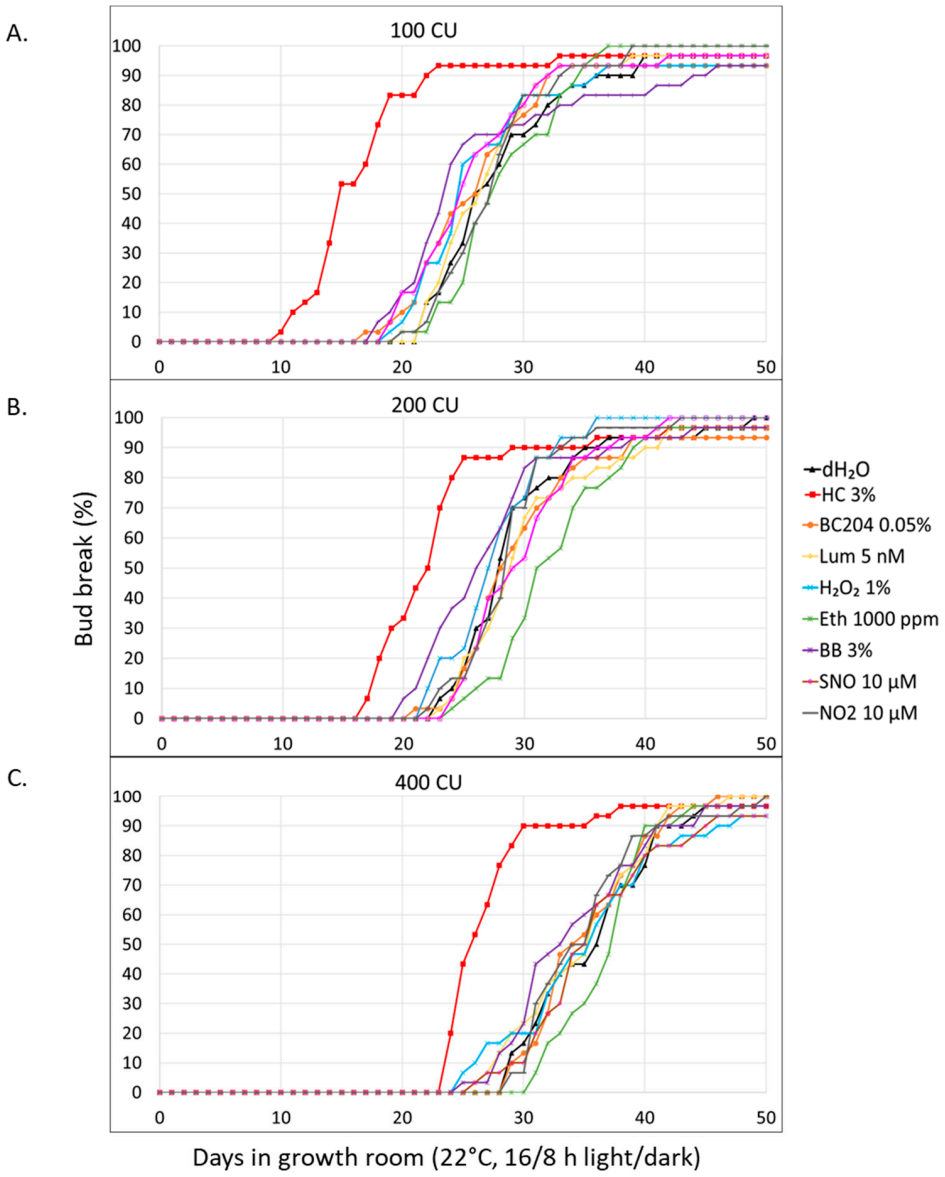

Figure 3. Effects of HC 3%, dH_2O, BC204 0.05%, Lum 5 nM, H_2O_2 1%, Eth 1000 ppm, BB 3%, SNO 10 µM, and NONOate (NO2) 10 µM on the bud-break of *V. vinifera* CS SNCs, after (**A**) 100, (**B**) 200, and (**C**) 400 CUA (30 biological replicates/treatment).

Bud-break rates of SNCs treated with dH_2O were −0.37, −0,46 and −0,31, and for HC 3%, −0.48, −0.48 and −0.71 after 100, 200 and 400 CUA, respectively (the larger negative the slope is, the steeper the increase in bud-break rate), (Table 3). The HC 3% treatment rates were significantly higher than the negative control after 100 and 400 CUA ($p = 0$). Additionally, compared to the negative control, buds treated with NONOate 10 µM consistently had significantly higher rates of −0.43, −0.54 and 0.39, respectively, in the order of increased CUA ($p = 0$ to 0.02). In addition to this, the rate of NONOate 10 µM after 200 CUA was significantly higher than that of HC 3% ($p = 0$). Some interesting trends were also observed in the EC_{50} results.

The EC_{50} values of HC 3%-treated SNCs after 100, 200 and 400 CUA were 15.51, 21.32, and 25.86, respectively (Table 3). Across all experiments, these were significantly the lowest EC_{50} values ($p = 0$). The EC_{50}s of the negative control SNCs were 26.82, 27.99 and 35.39, in the order of increased CUA. Two treatments, H_2O_2 1% and BB3%, had significantly lower EC_{50} values than dH_2O in all three experiments ($p = 0$ to 0.02), resulting in less days to reach 50% bud-break. These precise onset values after 100, 200 and 400 CUA were 24.77,

27.16, and 34.92 for H_2O_2 1%, and 23.79, 25.82 and 33.52 for BB 3%. Bud-break EC_{50} was reached earlier following BB 3% treatment compared to H_2O_2 1% in each case ($p = 0$). One treatment, Eth 1000 ppm, resulted in consistent delayed bud-break results compared to all other treatments.

Table 3. Respective coefficient estimates measured after HC 3%, dH_2O, BC204 0.05%, Lum 5 nM, H_2O_2 1%, Eth 1000 ppm, BB 3%, SNO 10 µM, and NONOate 10 µM application on *V. vinifera* CS SNCs after 100, 200, and 400 CUA (30 biological replicates/treatment). Treatments were assigned letters which indicate significant differences at 5% ($p < 0.05$) amongst the coefficient estimates. These letters only apply to individual experiments and parameters.

Bud-Break Parameter: Definition	Treatment	Coefficient Estimate		
		100	200	400
Onset: Number of days to first bud-break	HC 3%	9.86 ^{f}	16.12 ^{f}	25.41 ^{abcd}
	dH_2O	20.07 ^{a}	22.17 ^{b}	27.08 ^{b}
	BC204 0.05%	18.08 ^{c}	22.49 ^{bc}	27.58 ^{b}
	Lum 5 nM	20.47 ^{ab}	22.34 ^{bc}	25.91 ^{c}
	H_2O_2 1%	18.80 ^{d}	21.07 ^{d}	24.48 ^{d}
	Eth 1000 ppm	20.93 ^{b}	24.49 ^{a}	30.81 ^{a}
	BB 3%	16.48 ^{e}	19.16 ^{e}	25.17 ^{cd}
	SNO 10 µM	17.81 ^{c}	22.74 ^{bc}	27.36 ^{b}
	NONOate 10 µM	20.83 ^{b}	22.86 ^{c}	27.85 ^{b}
Rate: Slope between onset and final bud-break %	HC 3%	−0.48 ^{a}	−0.48 ^{ab}	−0.71 ^{a}
	dH_2O	−0.37 ^{b}	−0.46 ^{ac}	−0.31 ^{bc}
	BC204 0.05%	−0.34 ^{b}	−0.42 ^{dc}	−0.36 ^{de}
	Lum 5 nM	−0.45 ^{a}	−0.39 ^{d}	−0.30 ^{bc}
	H_2O_2 1%	−0.42 ^{a}	−0.45 ^{ac}	−0.25 ^{f}
	Eth 1000 ppm	−0.37 ^{b}	−0.38 ^{d}	−0.49 ^{g}
	BB 3%	−0.34 ^{b}	−0.38 ^{d}	−0.30 ^{b}
	SNO 10 µM	−0.36 ^{b}	−0.38 ^{d}	−0.33 ^{dc}
	NONOate 10 µM	−0.43 ^{a}	−0.54 ^{b}	−0.39 ^{e}
EC_{50}: Number of days to 50% of final bud-break %.	HC 3%	15.51 ^{g}	21.32 ^{h}	25.86 ^{f}
	dH_2O	26.82 ^{b}	27.99 ^{e}	35.39 ^{b}
	BC204 0.05%	25.43 ^{d}	28.37 ^{c}	34.80 ^{c}
	Lum 5 nM	26.07 ^{c}	28.82 ^{d}	34.60 ^{c}
	H_2O_2 1%	24.77 ^{e}	27.16 ^{f}	34.92 ^{c}
	Eth 1000 ppm	28.03 ^{a}	31.71 ^{a}	36.50 ^{a}
	BB 3%	23.79 ^{f}	25.82 ^{g}	33.52 ^{e}
	SNO 10 µM	24.98 ^{e}	29.25 ^{b}	34.93 ^{c}
	NONOate 10 µM	26.96 ^{b}	28.16 ^{ce}	34.26 ^{d}
Final percentage: Upper limit of cumulative bud-break % curve	HC 3%	95.95 ^{c}	96.40 ^{bc}	95.65 ^{e}
	dH_2O	95.81 ^{c}	97.48 ^{b}	100 ^{a}
	BC204 0.05%	96.85 ^{bc}	94.23 ^{e}	99.99 ^{ab}
	Lum 5 nM	96.35 ^{c}	95.27 ^{ec}	100 ^{a}
	H_2O_2 1%	92.61 ^{d}	99.94 ^{a}	98.19 ^{cd}
	Eth 1000 ppm	100 ^{a}	96.69 ^{bd}	97.10 ^{d}
	BB 3%	91.87 ^{d}	95.43 ^{ecd}	98.14 ^{cd}
	SNO 10 µM	97.77 ^{b}	99.38 ^{a}	95.55 ^{e}
	NONOate 10 µM	99.34 ^{a}	99.27 ^{a}	98.72 ^{cb}

The days it took SNCs treated with Eth 1000 ppm to reach onset were 20.93, 24.49 and 30.81 days after 100, 200 and 400 CUA, respectively (Table 3). Furthermore, SNCs treated with Eth 1000 ppm took 28.03, 31.71 and 36.50 days to reach EC_{50} after 100, 200 and 400 CUA, respectively. These onsets and EC_{50} values were significantly higher than those of the negative and positive controls ($p = 0$ to 0.02), excluding the onset of HC 3% after 400 CUA, in which case the values were similar. Treatments did not result in major

differences in the final bud-break %; however, there were still some significant differences seen in the datasets:

HC 3%-treated SNCs reached 95.95, 96.40 and 95.65 final bud-break %, whilst dH_2O -treated SNCs reached 95.81, 97.48 and 100 final bud-break % after 100, 200 and 400 CUA, respectively. In comparing the differences between these results, the higher % reached by dH_2O-treated SNCs after 400 CUA was the only significant difference ($p = 0$). Furthermore, there were only two treatments, SNO 10 µM and NONOate 10 µM, which resulted in significantly higher bud-break percentages than dH_2O in two of the three experiments ($p = 0$ to 0.01). These experiments included SNCs with 100 and 200 CUA. The final bud-break percentages of SNO 10 µM and NONOate 10 µM-treated SNCs were 97.77 and 99.34 after 100 CUA and 99.38 and 99.27 after 200 CUA, respectively. Nevertheless, all treatments across all three experiments resulted in final bud-break percentages above 90%.

3.2.2. Evaluation of Plant Biostimulants SN and PBX, as Well as Biochemical Agents Combined with Lumichrome and BC204

In 2022, the effects of the following treatments were evaluated on bud-break in *V. vinifera* CS SNCs after 100, 200 and 250 CU: HC 3%, dH_2O, MIX2, SNH, SNL, PBXH, and PBXL. This selection of treatments included two commercially available PBs, SN and PBX, and a combination of treatments tested in the previous year, which was named MIX2. The CU chosen for each experiment was based on assay optimisation from last year. Each treatment consisted of 45 biological replicates, and each experiment was performed in duplicate (data for second replicates not shown).

Fresh samples were collected for each experiment between mid-June and mid-July 2022 at approximately the final desired CU (Table S1). This procedure was implemented to avoid potential stress that may be introduced by long periods of exposure to artificial CUA. The results were that SNCs with higher CUA had more time to mature on the vines before sampling. Visualisations of cumulative bud break over time revealed several general trends for further consideration.

Bud-break across treatments generally started between 10 and 20 days after treatment, irrespective of final CUA (Figure 4). Additionally, the range of onset values became more condensed with increased CUA. The earliest onset date recorded in each experiment was approx. 10 days. This shows that stress introduced by artificial CUA was successfully avoided. After 100 CUA, HC 3% generally resulted in advanced bud-break, compared to other treatments; however, it increasingly caused phytoinhibition compared to the negative control, dH_2O, after 200 and 250 CUA. This is especially evident in the final bud-break percentage, which was close to 70 after 250 CUA. Most treatments generally resulted in cumulative bud-break curves with trends similar to that of the negative control across the three experiments. However, after 100 CUA, there were two exceptions, MIX2, and SNH, which had resulted in bud-break trends more similar to that of HC 3%. It was also noteworthy that SNH continued to produce bud-break curves similar in shape to that of HC 3% after 200 and 250 CUA. This included some phytoinhibitory results after treatment with SNH with higher CUA, particularly lower final bud-break %, which was around 80% after 250 CUA.

The negative control, dH_2O, resulted in 18.20, 15.48 and 13.77 days after 100, 200 and 250 CUA until bud-break onset was reached (Table 4). After HC 3% treatment, bud-break was generally initiated early across experiments, compared to the negative control and other treatments. For instance, the onset of HC 3% treated SNCs after 100 CUA was 9.69 days, which was significantly lower than all other treatments ($p = 0$). Compared to dH_2O, the onset of HC 3% treated SNCs was significantly lower after 200 CUA ($p = 0$), yet similar after 250 CUA, which were 10.35 and 13.72 days, respectively. Compared to HC 3%, one treatment, SNH, resulted in equal or reduced days to onset in two of the three experiments. Firstly, SNH resulted in a similar onset of 9.81 days after 200 CU and a significantly lower onset after 250 CUA, which was 10.14 days ($p = 0$). The latter also had the lowest onset compared to all treatments following 250 CUA.

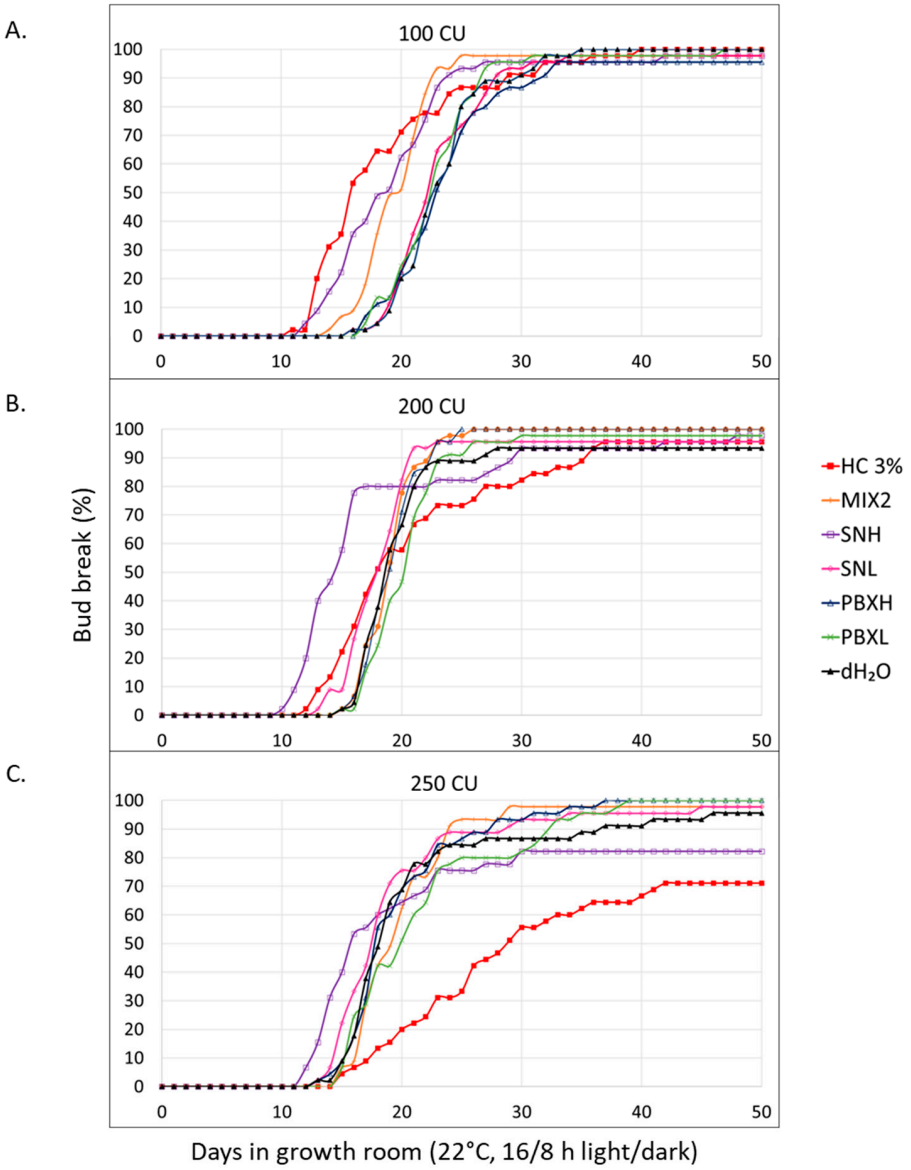

Figure 4. Effects of HC 3%, dH_2O, SNH, SNL, PBXH, PBXL and MIX2 on the bud-break of *V. vinifera* CS SNCs, after (**A**) 100, (**B**) 200, and (**C**) 250 CUA (45 biological replicates/treatment).

Considering the bud-break rate, HC 3%-treated SNCs had significantly the slowest rates, which were −0.32, −0.29 and −0.23 after 100, 200 and 250 CUA, respectively (Table 4). The bud-break rates of the negative control, which were −0.52, −0.79 and −0.59 after 100, 200 and 250 CUA, were the most rapid in each experiment, with one exception. This exception was recorded after 100 CUA, during which the MIX2 treatment resulted in the significantly highest rate of −0.62 compared to all treatments ($p = 0$). The EC_{50} values of each experiment provided further information on the effect of treatment on the rate of bud-break between treatments.

Generally, HC 3%-treated buds took longer to reach EC_{50} with increased CUA and were eventually surpassed by both the negative control and all treatments after 250 CUA. For instance, after 100 CUA, the EC_{50} value recorded after HC 3% treatment of 17.02 was significantly the least days ($p = 0$), and that of dH_2O, which was 22.88, was significantly the most days ($p = 0$) (Table 4). After 200 CUA, the EC_{50}s of HC-3%- and dH_2O-treated buds were 18.71 and 18.57 days, respectively, which were similar. Furthermore, after 250 CUA, HC 3%-treated SNCs took the most days, which was 24.96, to reach EC_{50} ($p = 0$). The 17.99 days it took buds treated with the negative control in this experiment was average

amongst treatments. Notably, after 200 and 250 CUA, SNH-treated SNCs significantly displayed the lowest EC_{50}s, which were 14.05 and 15.67, respectively ($p = 0$). After 100 CUA, this treatment resulted in an EC_{50} of 18.29, which was the second least, outcompeted only by HC% ($p = 0$).

Table 4. Respective coefficient estimates measured after HC 3%, dH_2O, SNH, SNL, PBXH, PBXL and MIX2 application on *V. vinifera* CS SNCs after 100, 200 and 250 CUA (45 biological replicates/treatment). Treatments have been assigned letters which indicate significant differences amongst the coefficient estimates. These letters only apply to individual experiments and parameters.

Bud-Break Parameter: Definition	Treatment	Coefficient Estimate		
		100	200	250
Onset: Number of days to first bud-break	HC 3%	9.69 ^{f}	10.35 ^{c}	13.72 ^{ab}
	dH_2O	18.20 ^{a}	15.48 ^{a}	13.77 ^{ac}
	MIX2	15.32 ^{d}	15.47 ^{a}	14.14 ^{a}
	SNH	11.67 ^{e}	9.81 ^{c}	10.14 ^{e}
	SNL	17.33 ^{b}	13.86 ^{b}	12.60 ^{bd}
	PBXH	16.76 ^{c}	15.50 ^{a}	13.20 ^{bc}
	PBXL	17.32 ^{b}	15.88 ^{a}	12.34 ^{d}
Rate: Slope between onset and final bud-break %	HC 3%	−0.32 ^{a}	−0.29 ^{a}	−0.23 ^{a}
	dH_2O	−0.52 ^{d}	−0.79 ^{b}	−0.59 ^{c}
	MIX2	−0.62 ^{b}	−0.83 ^{b}	−0.52 ^{bc}
	SNH	−0.39 ^{c}	−0.57 ^{c}	−0.44 ^{b}
	SNL	−0.50 ^{d}	−0.72 ^{bd}	−0.52 ^{bc}
	PBXH	−0.44 ^{e}	−0.77 ^{b}	−0.47 ^{b}
	PBXL	−0.52 ^{d}	−0.65 ^{cd}	−0.31 ^{d}
EC_{50}: Number of days to 50% of final bud-break %.	HC 3%	17.02 ^{e}	18.71 ^{bc}	24.96 ^{a}
	dH_2O	22.88 ^{b}	18.57 ^{c}	17.99 ^{f}
	MIX2	19.31 ^{c}	18.77 ^{bc}	19.09 ^{c}
	SNH	18.29 ^{d}	14.05 ^{e}	15.67 ^{g}
	SNL	22.35 ^{a}	17.64 ^{d}	17.46 ^{e}
	PBXH	22.83 ^{b}	18.89 ^{b}	18.57 ^{d}
	PBXL	22.40 ^{a}	19.81 ^{a}	20.18 ^{b}
Final percentage: Upper limit of cumulative bud-break % curve	HC 3%	98.15 ^{b}	93.25 ^{d}	70.71 ^{e}
	dH_2O	99.46 ^{a}	92.90 ^{d}	91.57 ^{c}
	MIX2	97.95 ^{b}	99.85 ^{a}	97.42 ^{a}
	SNH	97.29 ^{b}	92.65 ^{d}	81.03 ^{d}
	SNL	97.43 ^{b}	95.76 ^{c}	95.24 ^{b}
	PBXH	95.31 ^{c}	99.89 ^{a}	98.11 ^{a}
	PBXL	99.18 ^{a}	97.56 ^{b}	97.66 ^{a}

The final bud-break % of all the treatments across experiments resulted in values above 90%, except for HC 3% and SNH after 250 CUA (Table 4). The final bud-break percentage for HC 3% in this experiment was 70.71%, which was significantly the lowest ($p = 0$). This was followed by the 81.03% recorded after SNH treatment, which was significantly lower than the negative control and other experimental treatments ($p = 0$).

3.2.3. Small-Scale Field Trial: Evaluation of Plant Biostimulants SN and PBX

A small-scale field trial was started on the 6 September 2022, during which the effect of dH_2O, HC 3%, SNH and PBXH was evaluated. At the time of application, 627 CU had already accumulated in the *V. vinifera* CS block, which reached a maximum of 662 CU during the experiment (Figure 5). The cumulative bud-break plots show that HC 3% had resulted in a slightly delayed bud-break rate, whilst PBXH and SNH displayed results similar to that of the negative control.

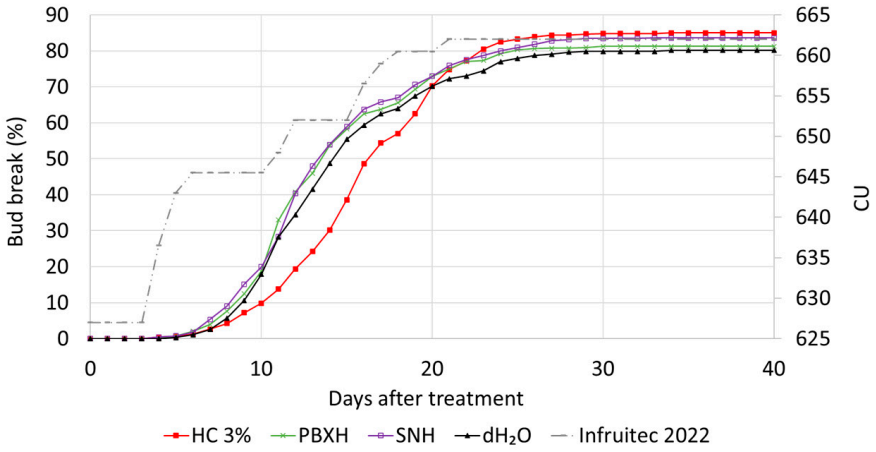

Figure 5. Effects of HC 3%, dH_2O, SNH, and PBXH on the bud-break of *V. vinifera* CS canes during the small-scale field trial.

Buds treated with the negative control took 6.53 days until bud-break was initiated, very similar to the 6.07 days it took PBXH buds to reach onset (Table 5). The treatment that significantly resulted in the least days to reach onset was SNH, which was achieved in 5.07 days ($p = 0$). Furthermore, the onset of buds treated with HC 3% was 8.38, which was significantly the longest time recorded for this parameter ($p = 0$). Nevertheless, in addition to resulting in the most days until onset, HC 3% treatment also resulted in the most days until EC_{50} was reached, which was 15.61 days. This is significantly longer than the 13.09, 12.62 and 12.74 days it took dH_2O and PBXH to reach EC_{50}, respectively ($p = 0$). However, HC 3% reached a final bud-break % of 85.26, which was significantly the highest among treatments ($p = 0$). The 80.20 and 82.28 final bud-break percentages reached by PBXH and SNH, respectively, were significantly higher than that of the negative control, dH_2O ($p = 0$). It is probable these buds are already in the ecodormant state so no differences can be distinguished between treatments.

Table 5. Respective coefficient estimates measured during *V. vinifera* CS dormancy-release small-scale field trial after HC 3%, dH_2O, SNH and PBXH treatment. Treatments have been assigned letters, which indicate significant differences amongst the coefficient estimates. These letters only apply to individual experiments and parameters.

Bud-Break Parameter: Definition	Treatment	Coefficient Estimate
Onset: Number of days to first bud-break	HC 3%	8.38 ^{a}
	dH_2O	6.53 ^{b}
	PBXH	6.07 ^{bc}
	SNH	5.70 ^{c}
Rate: Slope between onset and final bud-break %	HC 3%	−0.36 ^{a}
	dH_2O	−0.38 ^{a}
	PBXH	−0.39 ^{a}
	SNH	−0.38 ^{a}
EC_{50}: Number of days to 50% of final bud-break %.	HC 3%	15.61 ^{a}
	dH_2O	13.09 ^{b}
	PBXH	12.62 ^{c}
	SNH	12.74 ^{c}
Final percentage: Upper limit of cumulative bud-break % curve	HC 3%	85.26 ^{a}
	dH_2O	78.69 ^{d}
	PBXH	80.20 ^{c}
	SNH	82.28 ^{b}

4. Discussion

4.1. Forced Bud-Break Assays

4.1.1. Evaluation of Plant Biostimulants BC204, Lumichrome, and Candidate Biochemical Agents

The forced bud-break assays, which took place in 2021, evaluated the effects of two PBs, which were BC204 and lumichrome, as well as a selection of biochemical agents. The treatments included HC 3%, dH_2O, BC204 0.05%, Lum 5 nM, H_2O_2 1%, Eth 1000 ppm, BB 3%, SNO 10 µM, and NONOate 10 µM. Treatments were evaluated after insufficient CUA to reach a high-percentage bud-break (100 CU), sufficient CUA for a high-percentage bud-break (200 CU), and sufficient CUA to result in both a high-percentage and uniform bud-break (400 CU) [3,4,56,57]. All samples were collected on the same day and were thus all at the same level of maturity across experiments. Overall, the results of the 2021 forced bud-break assays demonstrated the relationship between HC and CUA and provided insight into possible mixture compositions that could lead to new formulations or alternative treatment strategies.

Overall, HC 3% advanced bud-break onset the most effectively; however, this became less distinct with higher CUA (Figure 3). Similarly, the bud-break rate was accelerated strongly by the HC 3% treatment, which was only outcompeted by 10 µM NONOate (NONOate 10 µM) after 200 CUA (Table 3). Interestingly, NONOate 10 µM was also the only treatment that resulted in higher bud-break rates than the negative control in all three experiments (Table 3). What was also noteworthy considering NO treatments, was that both NO donors in the current study, including NONOate 10 µM and 10 µM S-nitrosoglutathione (SNO 10 µM), resulted in enhanced final cumulative bud-break percentages in two of the three experiments (Table 3). Such results are in line with the literature, which has linked NO donors to bud dormancy-release, as well as its involvement in the HC-induced cascade [26,28].

In the current study, NO likely triggered hormone response in a similar fashion to HC [26–28]. For instance, it has been found that the exogenous application of NO donors, 8% (*w*/*v*) potassium nitrate (KNO_3) and 6% (*w*/*v*) calcium nitrate (Ca $[NO_3]_2$) increased gibberellic acid and indoleacetic acid levels and decreased that of ABA in dormant 'Anna' apple tree buds [58]. It was also reported that both donors hastened and improved the bud-break percentage compared to the negative control; however, these results were not superior to those of Dormex®. In agreement, it was shown that in 'Fuji' apple tree shoots, the bud-break enhancing abilities of 0.1–2 M KNO_3, and 0.05–1M sodium nitrite ($NaNO_2$), applied in combination with 3% (*v*/*v*) mineral oil were also not superior to that of 3% (*v*/*v*) Dormex® [32]. However, it was reported that the enzymatic generation of NO via a combination of $NaNO_2$ and ascorbic acid was, in some cases, equally effective to Dormex®. The product of this reaction directly generates NO and nitrites and does not rely on nitrite and/or nitrate reductases to produce NO. Indeed, this is a similar concept to using NO donors that release NO in a controlled manner once dissolved in water, as was performed in the present study, and supports the bud-break-enhancing abilities of NO in current results. Besides NO donors, which have shown potential regarding rate and final bud-break percentage, two other treatments, 1% H_2O_2 (H_2O_2 1%), and 3% BUDBREAK® mineral oil (BB 3%) (AECI Chemicals, Johannesburg, South Africa), showed potential in enhancing onset and EC_{50} values.

Despite HC 3% treatment reducing the number of days it took to reach bud-break onset and EC_{50} the most in each experiment, which was not surprising, H_2O_2 1% and BB 3% produced results superior to the negative control (Table 3). ROS (H_2O_2) and hypoxia, which can be created artificially with mineral oils such as BB3%, are clearly associated with dormancy-release in grapevine buds [26–28]. It was notable that BB 3% had produced slightly better results than 1% H_2O_2. Indeed, mineral and vegetable oils have been reported to enhance bud-break properties in woody perennial crops, such as grapevine and pear; however, they generally have not produced results superior to HC treatment [49,59,60]. This is in harmony with current results for BB 3%.

Compared to no treatment in 'Carmen' grapevine, 2% (*v*/*v*) vegetable oil and 2% (*v*/*v*) mineral oil have been found to increase bud-break percentage, both independently and in combination with one another, which agrees with current results [49]. Additionally, it was shown that the combination of the two oils enhanced the bud-break percentage in a similar manner to 3% (*v*/*v*) HC, which may be considered for future optimisation purposes. Furthermore, 2% (*v*/*v*) mineral oil was reported to lower CAT activity within 24 h after treatment, compared to the negative control. These results are supported by another study in Asian 'Hosui' pear trees, in which 4% mineral oil also inhibited CAT activity [60]. This may explain the lower onset and EC_{50} values displayed by grapevine buds treated with BB 3% in current results since the inhibition of CAT is known to occur under hypoxic conditions, which induces bud-break [26].

Hydrogen peroxide is a key signalling compound in the physiological regulation of natural dormancy release [28,61]. In agreement with current results, it has been shown that H_2O_2 may partially promote bud-break in perennial crops such as pear, grapevine, and walnut [24,61–64]. For example, in *V. vinifera* Thompson Seedless, 1% (*v*/*v*) H_2O_2 enhanced bud-break percentage compared to the negative control; however, it was not as effective as 2.5% (*w*/*v*) HC [63]. Furthermore, it was suggested that after application, H_2O_2 was rapidly degraded by CAT and did not result in activation of grape dormancy-breaking related protein kinase, which is known to be upregulated by HC [1,63]. In support of this, it was reported that the application of 1% (*v*/*v*) H_2O_2 to *V. vinifera*, Thompson Seedless buds that were in the stage of endo dormancy-release triggered genes encoding antioxidant enzymes, such as CAT [24].

The degradation by CAT may explain the inability of H_2O_2 treatment to produce results equal to that of HC in the current study. Additionally, it could explain the slightly inferior results compared to BB 3%, which likely activated anaerobic respiratory pathways that led to H_2O_2 accumulation and subsequent signalling [28]. It is known that H_2O_2 is a negative regulator of dormancy, which can even negatively affect bud-break and shoot growth when applied in too high concentrations or when buds have matured past the stage of endodormancy [61]. However, this may not have been the case in current results as 1% (*v*/*v*) H_2O_2 has been tested for bud-break by other research studies in grapevine, which reported similar trends in results [24,63].

Ethylene treatment using 0.206% *v*/*v* Ethephon®, an ethylene supplement (Eth 1000 ppm), resulted in prolonged bud-break compared to HC. Eth 1000 ppm-treated SNCs consistently took the greatest number of days to reach both bud-break onset and EC_{50} (Table 3). Indeed, it has been reported that the exogenous application of 500 and 1000 ppm ethephon, which is similar to the treatment concentration applied in the present study, prolonged dormancy-release in 'Muscat of Alexandria' grapevine plants grown in a greenhouse [65]. However, such results contrast reports that 100 ppm ethylene enhanced the bud-break of *V. vinifera* 'Perlette' cuttings [19]. In addition to this, another study on *V. vinifera* Thompson Seedless found that the exogenous application of 10 mg ethephon, dissolved in 10 mL 100 mM phosphate buffer, upregulated genes involved in oxidative stress responses, which is associated with bud-break [24]. Thus, the ability of ethylene supplementation to enhance bud-break traits may be subjected to optimal application concentrations.

4.1.2. Evaluation of Plant Biostimulants SN and PBX, as Well as Biochemical Agents Combined with Lumichrome and BC204

Forced bud-break assays, which took place in 2022, evaluated the effects of two controls, which were HC 3% and dH_2O, two commercial PBs, which were SN and PB, and MIX2, which was a combination treatment consisting of treatments tested in the previous year, after 100, 200 and 250 CUA (Table 2). All treatments resulted in high final bud-break percentages, except for HC 3% and SNH after 250 CUA (Figure 4) [3,4,56,57]. The fact that HC 3% had displayed increased phytoinhibition at higher CUA was unexpected as this did not occur in the forced bud-break assays of 2021, during which buds had accumulated up

to 400 CU, which is much higher than the maximum of 250 CUA in these results (Figure 3). A possible explanation for this phenomenon is that the SNCs for all experiments in the previous year were collected on the 31 May, which at the time had accumulated only 14.3 to 50% of final CUA. In contrast, SNCs in current experiments were collected individually for each experiment on slightly later dates, 10 June to 12 July, and had accumulated 80 to 95% of the final CUA. Buds in the later experiments were thus allowed a longer time to age on the vine.

The reasoning behind the strategy of individual sampling per experiment was to bypass any stress introduced during artificial cold accumulation in 2022. Seemingly, this was successful, with no overall delay in onset between experiments in 2022, as seen in the previous year (Figure 4). However, the introduction of variation in bud maturity should be considered in future studies. Indeed, the optimal timing of application of forced bud-break agents such as HC is vital, and early application may cause uneven bud-break. In contrast, late application risks bud damage due to phytoinhibition or other stress factors [66]. Considering this, it is most likely that after 250 CUA, the buds were in a metabolic state unsuitable for HC treatment.

One of the most noteworthy outcomes was that after 250 CUA, SNH treatment had negatively affected final bud-break %, in a similar manner, albeit to a lesser extent compared to HC 3% (Figure 4). Cumulative bud-break percentage plots of SNCs treated with SNH were also quite similar in shape to those after HC 3% treatment. These results suggest that SNH may have induced similar physiological changes within dormant buds to HC, whilst being less phytoinhibitory than HC, or simultaneously providing nutrients, which aids in stress response and cell recovery. Indeed, chemical and physical methods other than HC that are known to induce bud-break, including heat shock, sodium azide, and hypoxia, have been found to simulate the pathways induced by HC in grapevine [19,24,26,58]. Besides the bud-break percentage, the onset and EC_{50} values of HC 3% and SNH were superior to other treatments and the negative control (Table 4). Furthermore, SNH treatment generally accelerated the onset and EC_{50}.

SNH consists of two synergistically acting substances, including 2% (*v*/*v*) SBB-01 and 20% (*v*/*v*) NDY-01 (code names were supplied by manufacturer/supplier). Both SBB-01 and NDY-01 are rich in various forms of nitrogen. Additionally, SBB-01 is an amino acid-based biostimulant, and NDY-01 contains water-soluble calcium (CaO), which could enhance Ca signaling similarly to HC [26]. Indeed, the most common agrochemicals evaluated for dormancy release are amino acid and nitrite salt-based [37]. The performance of such agrochemicals has been tested in crops such as kiwi, apple, sweet cherry, grapevine, blackberry, and peach [25,30,33–36,67,68]. Generally, treatments showed enhancing effects on bud-break and, in some cases, resulted in similar or superior results compared to HC, which are aligned with current results for SNH. For instance, in 'Gala' and 'Fuji' apple trees, it was demonstrated that Erger®, Synchron® and Vorax® in combination with mineral oil or calcium nitrate, can all effectively enhance axillary and terminal bud-break percentages [25]. Overall results varied compared to HC treatment, with Synchron® performing better, Erger® similarly, and Vorax® worse than HC. In another study, Erger® application with calcium nitrate was tested in the 'Fiano' grapevine cultivar, during which 3, 5 and 7% (*v*/*v*) Erger® enhanced bud-break compared to the control, but not compared to 4% (*v*/*v*) Dormex® [35]. Nevertheless, one treatment, which was MIX2, in the current study showed the potential to enhance the bud-break rate.

Bud-break rates of the negative controls were not better than HC 3%, nor any other treatment across experiments, except MIX2 after 100 CU. This treatment produced a notable result. MIX2 treatment probably introduced hypoxia, as well as ROS and NO signalling. This may have been due to the inclusion of BB 3%, H_2O_2 1%, NONOate 10 µM and SNO 10 µM in the treatment. The relevance of these individual treatments to dormancy release has previously been explored. However, two PBs were also included in MIX2, which were 5 nM lumichrome and 0.05% (*v*/*v*) BC204.

All the components of MIX2 were evaluated independently during the 2021 forced bud-break assays, all of which had shown some potential in enhancing bud-break, except for the two PBs. Nevertheless, a synergistic interaction between two or more components may have been at play, which ultimately enhanced bud-break rate and/or stress tolerance in the SNCs. In addition to activating anaerobic metabolism, mineral oil prevents evaporation and ensures the penetration of treatments into plant tissue [53]. The mineral oil likely improved the uptake of the other ingredients in the treatment. Collectively, interference with aerobic respiration, as well as NO and ROS signalling, are likely to enhance the bud-break rate in a similar fashion to the way HC works on dormant buds [26–28].

The two PBs may have promoted growth in the presence of oxidative stress introduced by the other ingredients of MIX2. Their enhanced performance in the current treatment may have been explained by their application and BB3%, which may have improved treatment uptake. BC204, which is currently being used for colour enhancement on various crops, including CS table grapes, has been found to increase crop yield, fruit quality, plant health and stress response [52]. For example, 0.01% (*v*/*v*) BC204 enhanced *Arabidopsis thaliana* growth, even in the presence of osmotic stress introduced by 100 mM NaCl. An RNA-seq study showed that in *A. thaliana* shoot tissue, BC204 differentially affected genes related to dormancy release, such as cell wall synthesis, carbon metabolism, transcription factors, calcium regulation and phytohormones [52]. Furthermore, 5 nM lumichrome increased photosynthesis and enhanced plant growth via turgor-driven cell expansion (upregulation of *XTH9* and *XPA4*) in *A. thaliana* [51]. However, these present data indicate that BC204 and lumichrome do not seem to contribute to dormancy release in grapevine buds. However, they may be found to enhance the growth and general health of the shoots that emerge post-bud-break upon further evaluations.

4.2. Small-Scale Field Trial: Evaluation of Plant Biostimulants SN and PBX

In 2022, a small-scale field trial was conducted, during which the effects of HC 3%, dH_2O, SNH, and PBXH on bud-break were evaluated. Treatments were applied approx. Two weeks before predicted bud-break, in accordance with commercial guidelines and pruning schedules based on grower advice. The Berg River region, where the trial was conducted, may simply not have been the optimal location to conduct bud-break trials, considering high numbers of CU (627 CUA) that had accumulated by the time that the trial was initiated (Figure 5). This is well above the required CU for high and even bud-break, which eliminated the need for artificial dormancy-release [3,4,56,57].

However, using HC locally in the Paarl area is implemented as a routine treatment amongst most growers. Nonetheless, HC 3% treated buds took the longest to reach EC_{50}, suggesting that HC may negatively affect bud-break uniformity when applied to buds already receiving sufficient CU (Table 5) [66]. However, HC 3% achieved the highest final bud-break % among all treatments. The two PBs displayed qualities similar to that of water. However, future more expansive controlled field trials are necessary to obtain more robust and accurate datasets.

4.3. On the Development of Novel Treatments: Summary Model of Key Components

Alternative dormancy-release treatments typically consist of the following three ingredients, which should be considered in the development of future treatments (Figure 6):

1. A source of hypoxia, such as an oil-based adjuvant or mineral/vegetable oil
2. Inclusion of additional supplementation of nitrites and/or nitrates such as potassium nitrite (KNO_2) or KNO_3
3. Amino acids

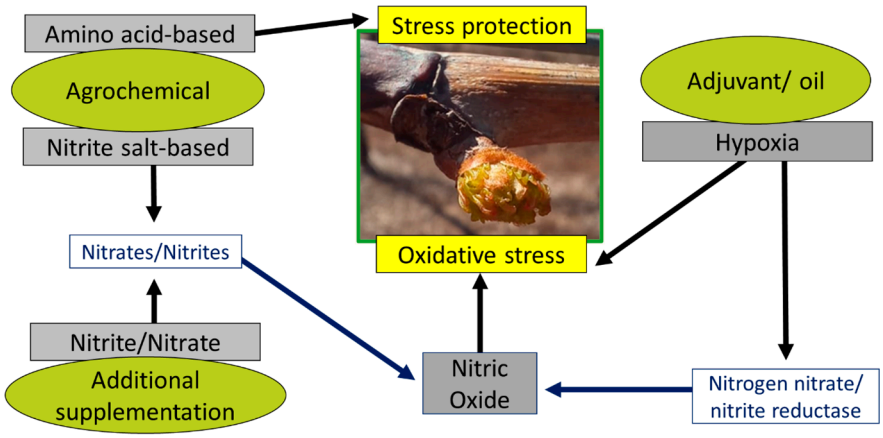

Figure 6. The components typically present in alternative dormancy-release treatments, which are agrochemical and biochemical agent based. Nitric oxide donors, and hypoxia create oxidative stress, which act as central signalling mechanisms for dormancy-release. Amino acid supplementation is used by the plant to produce compounds which aid in protection against uncontrolled reactive oxygen accumulation and recovery.

Together, the abovementioned components should ideally induce dormancy-release and aid in cell recovery in the following ways. Oxidative stress induced by the candidate alternative PB-based agrochemical treatments is likely attributed lead to the formation of respiratory stress and NO from nitrites and nitrates enzymatically via the activation of nitrogen nitrite and nitrite reductases under hypoxic conditions. NO is a type of ROS, which both inhibits cytochrome oxidase and has been shown to inhibit CAT in a reversible manner, which further contributes to the spike in ROS [43,44]. Hypoxic conditions are artificially created by commercial rest breaking oils or oil-based adjuvants. Additionally respiratory stress, which is induced by rest breaking oils leads to ROS accumulation, such as H_2O_2, which is a natural trigger for various processes involved in dormancy-release [28]. Amino acids aid in stress response and recovery:

Changes in amino acid profiles are associated in plants which are under major oxidative stress, and the exogenous application of several amino acids are known to improve antioxidant defence [46]. Protein degradation, which occurs because of oxidative stress, results in the release of amino acids, which are used by the plant to biosynthesise various protective metabolites such as osmolytes, secondary metabolites, structural components needed for cell wall repair, hormones, polyphenols, cyanogenic glycosides, glucosinolates, and acyl sugars.

5. Conclusions

Rising global winter temperatures due to climate change are an increasing threat to agricultural practices globally, continuously introducing abiotic stress at a pace too rapid to allow for evolution and adaption [69]. Average winter temperatures have increased annually and are predicted to continue rising all over the world, including grape-producing regions in South Africa [8,13,70,71]. This is in accordance with the lower CUA accumulated in 2022 than in 2021 in the experimental site, which was in the Berg River table grape-producing region of South Africa (Figure S3). Despite sufficient CUA in this region, Dormex® is typically used as a preventative measure. Its use may soon become unavoidable, which is already the case for most of the remaining South African table grape-producing regions, including the Berg-, Olifants- and Orange River regions, and the Northern Regions. This emphasizes the importance of research results aimed at discovering an alternative product, such as those obtained in the current study.

The results of the current study highlighted several key elements which should be included in future candidate HC replacement treatments. The results obtained during the 2021 forced bud-break assays indicated that treatment of dormant grapevine compound buds with

NO, H_2O_2, and hypoxia do indeed trigger dormancy release to a certain extent, supporting the molecular models proposed for HC action [26,27]. Furthermore, during the 2022 forced bud-break assays, it was shown that NO, H_2O_2, and hypoxia, in combination with PBs, may potentially replace HC, provided the correct application strategy is used. However, this needs to be confirmed in future experiments. The relevance of amino acid and nitrogen-salt-based agrochemicals that induce oxidative stress was also confirmed by the results recorded after SNH treatment. These products might be best applied with an adjuvant and/or dormancy oil to enhance uptake and performance in future studies [53,59,72]. Finally, in 2022, the complexity of field trials compared to controlled glasshouse conditions and the need for optimal timing of HC application were emphasized and require further exploration.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050471/s1, Figure S1: Example of growth room temperatures during glasshouse assays; Figure S2: Visual representation and definitions of four parameters used to assess bud-break efficacy; Figure S3: Infruitec units accumulated in the *V. vinifera* CS block on Windmeul farm. Table S1: Summary of experiment dates and CU information for experiments evaluating the effects of HC 3%, MIX2, SNH, SNL, PBXH, PBXL, and dH_2O, in *V. vinifera* Crimson Seedless (CS) buds after, 100, 200 and 250 CUA.

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